IN AF


HAMILTON
BROOK
SMITH &
REYNOLDS, P.C.

PATENTS, TRADEMARKS
COPYRIGHTS & LITIGATION

530 VIRGINIA ROAD
P.O. BOX 9133
CONCORD, MA 01742-9133
TEL (978) 341-0036
FAX (978) 341-0136
www.hbsr.com



MUNROE H. HAMILTON
(1906-1984)

DAVID E. BROOK
JAMES M. SMITH
JOHN L. DUPRÉ
DAVID J. BRODY
MARY LOU WAKIMURA
ALICE O. CARROLL
N. SCOTT PIERCE
SUSAN G. L. GLOVSKY
DOREEN M. HOGLE
DAVID J. THIBODEAU, JR.
ANNE J. COLLINS
TIMOTHY J. MEAGHER
STEVEN G. DAVIS
DEIRDRE E. SANDERS
MARY K. MURRAY
MARK B. SOLOMON
BRIAN T. MORIARTY*

THOMAS J. ARRIA
MICHAEL J. BADZINSKI
SUMEDHA A. BAHRI
SETH M. CANNON
JEAN-PAUL CASS
MARIA CEDRONI
H. JOON CHUNG
F. JAMES COE
KRISTIN A. CONNARN
CHRISTINE M. DOE
COLIN C. DURHAM
CAROL A. EGNER
GIOVANNA FESSENDEN
JERRY KAZANJIAN
KEVIN T. SHAUGHNESSY
ROBERT H. UNDERWOOD
KEITH J. WOOD
MICHAEL M. YAMAUCHI

OF COUNSEL
LEO R. REYNOLDS
ELIZABETH W. MATA
DARRELL L. WONG

PATENT AGENTS
SUSAN M. ABELLEIRA
ALEXANDER AKHIEZER
KAMILAH ALEXANDER
ERIC M. BALICKY
MICHAEL GOTTSELIG
SUSAN C. KELLY
VIVIEN J. TANNOCH-MAGIN
PAMELA A. TORPEY

TECHNOLOGY SPECIALISTS
JOHN T. HURLEY
SARAH J. RODRIGUEZ
BOOYONG SHIM LIM
BENJAMIN J. SPARROW
CHRISTIANA ZHANG

MICHAEL KEWESHAN
ADMINISTRATIVE DIRECTOR

BARBARA J. FORGUE
ADMINISTRATOR OF
PATENT AND
TRADEMARK PRACTICE

*NOT ADMITTED IN MA



OIPE IAP17
AUG 16 2006
PATENT & TRADEMARK OFFICE


CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450

on August 14, 2006 Donna Boudreau
Date Signature

Donna Boudreau
Typed or printed name of person signing certificate

August 14, 2006

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

| | | |
|---|---|---|
| Re: | Appellant: | Michael J. Briskin |
| | Application No.: | 08/875,849 Filed: September 8, 1997 |
| | Confirmation No.: | 4411 |
| | Title: | MUCOSAL VASCULAR ADDRESSINS AND USES THEROF |
| | Docket No.: | 1855.1004-002 |

Sir:

Transmitted herewith is an Amended Appeal Brief for filing in the subject application. The Amended Appeal Brief is filed pursuant to the Notice of Non-Compliant Appeal Brief mailed from the U.S. Patent and Trademark Office on July 13, 2006.

1. [ ] Appellant hereby petitions to extend the time for filing an Appeal Brief for [ ] month(s) from [ ] to [ ].

2. [ ] A [ ] month extension of time to extend the time for filing an Appeal Brief from [ ] to [ ] was filed on [ ] with payment of a $[ ] fee.

   [ ] Appellant hereby petitions for an additional [ ] month extension of time for filing an Appeal Brief from [ ] to [ ].

3. [ ] A Request for Oral Hearing before the Board of Patent Appeals and Interferences is being filed concurrently herewith.

4. Fees are submitted for the following:

| | | | | |
|---|---|---|---|---|
| [ ] | Extension of Time for [ ] month(s) | | | $ ______ |
| [ ] | Additional Extension of Time: | | | |
| | Fee for Extension | ([ ] mo.) | $ ______ | |
| | Less fee paid | ([ ] mo.) | - $ ______ | |
| | Balance of fee due | | | $ ______ |
| [ ] | Appeal Brief | | | $ ______ |
| [ ] | Other ______ | | | $ ______ |
| | | | TOTAL | $ 0 |

5. The method of payment for the total fees is as follows:

[ ] A check in the amount of $[ ] is enclosed.

[ ] Please charge Deposit Account No. 08-0380 in the amount of $[ ].

Please charge any deficiency or credit any overpayment in the fees that may be due in this matter to Deposit Account No. 08-0380. A copy of this letter is enclosed for accounting purposes.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Robert H. Underwood

Robert H. Underwood
Registration No.: 45,170
Telephone: (978) 341-0036
Facsimile: (978) 341-0136

Concord, MA 01742-9133
Dated: August 14, 2006

@PFDesktop\::ODMA/MHODMA/HBSR05;iManage;642872;1
DES/RHU
August 11, 2006

PATENT APPLICATION
Docket No.: 1855.1004-002

O I P E
AUG 16 2006
PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| Appellant: | Michael J. Briskin | | |
| Application No.: | 08/875,849 | Group: | 1644 |
| Filed: | September 8, 1997 | Examiner: | R. Schwadron, Ph.D. |
| Confirmation No.: | 4411 | | |
| For: | MUCOSAL VASCULAR ADDRESSINS AND USES THEREOF | | |

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450
on August 14, 2006 Donna Boudreau
Date Signature
Donna Boudreau
Typed or printed name of person signing certificate

## AMENDED APPEAL BRIEF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This Amended Appeal Brief is submitted pursuant to the Notice of Non-Compliant Appeal Brief mailed from the U.S. Patent and Trademark Office (USPTO) on July 13, 2006. The Appeal Brief filed on April 21, 2006, pursuant to the Notice of Appeal received in the USPTO on September 19, 2005, is considered to be non-compliant because the Claims Appendix contains copies of withdrawn claims 101, 117 and 151, and because the arguments refer to claim 151. This Amended Appeal Brief is filed in support of the appeal from the rejections set forth in the Office Action mailed on August 15, 2005.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

The real party in interest is Millennium Pharmaceuticals, Inc., a corporation organized and existing under the laws of the State of Delaware, and having a usual place of business at 40 Landsdowne Street, Cambridge, Massachusetts 02139. Millennium Pharmaceuticals, Inc. is the Assignee of the entire right, title and interest in the subject application, by virtue of an Assignment recorded on September 19, 1997, at Reel 8713, Frames 0837-0841, and a merger recorded on June 8, 2000, at Reel 010907, Frames 0952-0955.

II. RELATED APPEALS AND INTERFERENCES

The Board's attention is directed to the appeal in U.S. Patent Application No. 08/523,004.

III. STATUS OF CLAIMS

Claims 24-26, 28-32, 101, 105-108, 111-113, 115-121 and 124-160 are pending.

Claims 1-23, 27, 33-100, 102-104, 109, 110, 114, 122 and 123 were canceled. Claims 101, 117 and 151 are withdrawn from consideration as being drawn to non-elected species. Claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124-150, and 152-160 are rejected.

Claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124-150, and 152-160 are on appeal. A copy of the appealed claims appears in the Claims Appendix of this Brief.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the rejections in the Office Action mailed August 15, 2005.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The invention relates to fusion proteins that comprise a primate Mucosal Addressin Cell Adhesion Molecule (MAdCAM) moiety. MAdCAM is a glycoprotein that is preferentially expressed on the surface of vascular endothelial cells lining blood vessels in the gastrointestinal tract. MAdCAM binds to the $\alpha 4\beta 7$ integrin, which is expressed on the surface of some lymphocytes, and participates in the homing of lymphocytes to mucosal tissues. Specification at

page 4, line 25 through page 5, line 4. The primary structure of two species of naturally occurring human MAdCAM are disclosed in Figures 1 and 2, and the primary structure of one species of naturally occurring macaque MAdCAM is disclosed in Figure 3. Id. at Figures 1-3, and SEQ ID NOS:2, 4 and 6.

In some aspects, the invention is a fusion protein that comprises a naturally occurring primate MAdCAM that binds α4β7 integrin, such as a naturally occurring human MAdCAM that binds α4β7 integrin. Id. at page 14, lines 18-28, page 15, line 22 through page 17, line 17, and page 13, lines 3-29.

In particular aspects (claims 24 and 136), the fusion protein comprises a naturally occurring primate MAdCAM, wherein said naturally occurring primate MAdCAM binds α4β7 integrin and has at least about 75% amino acid sequence similarity, or at least about 90% amino acid sequence similarity, to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6. Id. at page 17, lines 23-29.

In other particular aspects (claims 113 and 149), the fusion protein comprises a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds α4β7 integrin and has at least about 75% amino acid sequence similarity, or at least about 90% amino acid sequence similarity, to SEQ ID NO:2 or SEQ ID NO:4. Id. at page 17, lines 23-29.

In other aspects, the invention is a fusion protein that comprises an α4β7 integrin-binding fragment of a naturally occurring primate MAdCAM, such as an α4β7 integrin-binding fragment of a naturally occurring human MAdCAM. Id. at page 13, lines 3-15, and page 14, lines 18-28.

In particular aspects (claim 107), the fusion protein comprises an α4β7 integrin-binding fragment of a naturally occurring primate MAdCAM, wherein said primate MAdCAM has at least about 75% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, and said α4β7 integrin-binding fragment comprises the N-terminal immunoglobulin-like domain of said primate MAdCAM. Id. at page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other particular aspects (claim 145), the fusion protein comprises an α4β7 integrin-binding fragment of a naturally occurring primate MAdCAM, wherein said naturally occurring primate MAdCAM has at least about 90% amino acid sequence similarity to SEQ ID NO:2, SEQ

ID NO:4 or SEQ ID NO:6, and said $\alpha4\beta7$ integrin-binding fragment comprises at least one immunoglobulin-like domain of said primate MAdCAM. Id. at page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other particular aspects (claim 120), the fusion protein comprises an $\alpha4\beta7$ integrin-binding fragment of a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds $\alpha4\beta7$ integrin and has at least about 75% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4, and said $\alpha4\beta7$ integrin-binding fragment comprises the two N-terminal immunoglobulin-like domains of said human MAdCAM. Id. at page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other particular aspects (claim 154), the fusion protein comprises an $\alpha4\beta7$ integrin-binding fragment of a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4, and said $\alpha4\beta7$ integrin-binding fragment comprises the two N-terminal immunoglobulin-like domains of said human MAdCAM. Id. at page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other particular aspects (claim 157), the fusion protein comprises a primate MAdCAM or $\alpha4\beta7$ integrin-binding fragment thereof, wherein said primate MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4, and said $\alpha4\beta7$ integrin-binding fragment comprises at lease one immunoglobulin-like domain of said primate MadCAM. Id. at page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other aspects, the invention is a fusion protein comprising a primate MAdCAM moiety that has binding affinity for $\alpha4\beta7$ integrin.

In particular aspects (claim 126), the fusion protein comprises a primate MAdCAM moiety, wherein said primate MAdCAM moiety has binding affinity for $\alpha4\beta7$ integrin and comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2 and the

amino acid sequence of an $\alpha4\beta7$ integrin-binding portion of the polypeptide shown in Figure 1 (SEQ ID NO:2), wherein said $\alpha4\beta7$ integrin-binding portion comprises the N-terminal immunoglobulin-like domain. Id. at page 15, lines 21 through page 17, line 17, page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

In other particular aspects (claim 131), the fusion protein comprises a a primate MAdCAM moiety, wherein said primate MAdCAM moiety has binding affinity for $\alpha4\beta7$ integrin and comprises an amino acid sequence selected from the group consisting of SEQ ID NO:4 and the amino acid sequence of an $\alpha4\beta7$ integrin-binding portion of the polypeptide shown in Figure 2 (SEQ ID NO:4), wherein said $\alpha4\beta7$ integrin-binding portion comprises the N-terminal immunoglobulin-like domain. Id. at page 15, lines 21 through page 17, line 17, page 17, lines 23-29, page 16, lines 25-31, Figure 6, page 19, lines 11-19, page 20, line 20 through page 21, line 12, and page 72, line 19 through page 73, line 6.

## VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 24-26, 28-32, 103, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 and 152-160 are properly rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that is not supported by adequate written description.

B. Whether claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 and 152-160 are properly rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to make or use the invention.

C. Whether claims 24-26, 28-31, 105-108, 111, 113, 115, 116, 118, 120, 121, 124, 126-142, 144-147, 149, 150, 152, 154, 155 and 157-160 are properly rejected under 35 U.S.C. § 103(a) as being obvious over Butcher *et al.* (WO 94/13312, Reference AD of record) in view of Vonderheide *et al.* (U.S. Patent No.

5,599,676, Reference AB or record) and Erle *et al.* (*J. Immunol. 153*:517-528 (1994); Reference AX3 of record).

D. Whether claims 32, 112, 119, 125, 143, 148, 153 and 156 are properly rejected under 35 U.S.C. § 103(a) as being obvious over Butcher *et al.* (WO 94/13312, Reference AP of record) in view of Vonderheide *et al.* (U.S. Patent No. 5,599,676, Reference AB of record) and Erle *et al.* (*J. Immunol. 153*:517-528 (1994); Reference AX3 of record), and further in view of Capon *et al.* (U.S. Patent No. 5,656,335; Reference AF or record)

## VII. ARGUMENT

### A. CLAIMS 24-26, 28-32, 103, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 AND 152-160 ARE NOT PROPERLY REJECTED UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, AS CONTAINING SUBJECT MATTER THAT IS NOT SUPPORTED BY ADEQUATE WRITTEN DESCRIPTION.

At paragraph 3 of the Office Action dated August 15, 2005 ("Office Action"), it is stated that claim 103 is subject to rejection. However, claim 103 has been canceled. The Examiner is requested to clarify the record by indicating that claim 103 is not subject to rejection in the Examiner's Answer.

The rejection of record reflects the Examiner's opinion that the claimed subject matter is not supported by adequate written description. It is acknowledged that the application discloses the amino acid sequence of macaque MAdCAM and the amino acid sequences of two different forms of human MAdCAM. Office Action at 2. However, the Examiner concludes that written description is lacking because, with the exception of the disclosed sequences, the skilled person could not envision the detailed structure of the encompassed proteins. Id. Two rationales for the rejection are set forth in the Office Action. Id. at 5-6.

The first rationale focuses on the scope of the genus of primate MAdCAM proteins, and the Examiner states that the application discloses "a minuscule fragment of the potential

MAdCAMs derived from species encompassed by the term primate." Id. at 5. The Examiner also states that the application does not disclose "how many of the known primate sequences would be encompassed by the percent similarity language recited in the claims." Id.

The second rationale focuses on the structure and function of the MAdCAMs and binding fragments recited in the claims. In this regard, the Examiner states that "there is no disclosure as to what particular amino acids [sic] substitutions could be tolerated in any particular section of the sequence with the retention of MAdCAM function." Id. at 5-6. The Examiner further states that none of the claims recite 95% sequence identity and, therefore, that Example 14 of the Synopsis of Application of Written Description Guidelines (available on line at www.uspto.gov/web/offices/pac/writtendesc.pdf; "Application of Guidelines") is not germane to whether the claims are supported by adequate written description. Office Action at 4.

1. The rejection should be reversed, because the specification contains a written description of the claimed subject matter that is sufficient to show possession of the claimed subject matter under the standard set forth in the case law and in the PTO Guidelines.

The written description requirement is satisfied when the specification describes the claimed invention in sufficient detail so that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Each case must be decided on its own facts, and the precedential value of cases deciding the issue of written description is limited. Id. at 1562, 19 USPQ2d 1116. However, the case law provides well-established legal guidance for evaluating the facts of each case. This legal guidance has been incorporated into the Guidelines for the Examination of Patent Applications Under 35 U.S.C. § 112, para. 1, "Written Description" Requirement. MPEP § 2163 (8th ed., Rev. 3, Aug. 2005 ("Guidelines")).

The U.S. Court of Appeals for the Federal Circuit has adopted the PTO standard, articulated in the Guidelines, as persuasive authority for determining compliance with the written description requirement for an invention that is described functionally. Enzo Biochem Inc. v. Gen-Probe Inc., 296 F.3d 1316, 1324, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002) ("We are

persuaded by the Guidelines on this point and adopt the PTO's applicable standard for determining compliance with the written description requirement.").

Possession of the invention can be shown by "describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention." Guidelines at 2100-165. "The description need only describe in detail that which is new or not conventional." Id. at 2100-171. "An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics which provide evidence that applicant was in possession of the claimed invention, *i.e.*, complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." Id. at 2100-165. The PTO has published materials that illustrate application of the Guidelines. Application of Guidelines.

In view of the foregoing legal standard for assessing adequacy of written description, the Examiner's focus on the scope of the genus of primate MAdCAM proteins, and statements that the application discloses "a minuscule fragment of the potential MAdCAMs derived from species encompassed by the term primate," and that the application does not disclose "how many of the known primate sequences would be encompassed by the percent similarity language recited in the claims," are misplaced. Office Action, at 5.

Although the scope of the claims is relevant to the determination of whether the application contains adequate written description of the claimed subject matter, the focus is on the claimed subject matter with all of the claimed limitations. The focus should not be on the number of animal species that might be considered to be primates. In addition, it is unclear precisely what the Examiner's concern is regarding whether "known primate sequences" are encompassed by the claims. First, it is apparent from the prosecution history of this application that there were no "known primate sequences" at the time the application was filed, because none of the claims are rejected under 35 U.S.C. § 102. Thus, it appears that the Examiner may be concerned that a person skilled in the art would not be able to determine whether a particular amino acid sequence of a MAdCAM protein is encompassed by the claims. However, this should not be of concern, because the skilled person would merely align the sequence in question and SEQ ID NO:2, 4 or 6 to determine the percentage of amino acid sequence similarity between

the sequences using the method and parameters disclosed in the specification. Id. at 48, lines 26-31.

With respect to the Examiner's statement concerning the alleged lack of teachings of which amino acids are important for $\alpha4\beta7$ integrin binding function, Appellant directs the Board's attention to the extensive disclosure and exemplification of the application, which are set forth below.

a. The extensive description and exemplification in the application is sufficient to show possession of the claimed fusion proteins.

The application discloses the claimed subject matter in a manner that is sufficient to show that the inventor had possession of the invention at the time the application was filed. The disclosure of the application includes the description and exemplification of three species of naturally occurring primate MAdCAM, a detailed description of the structure of naturally occurring primate MAdCAM, a disclosure of the relationship between structural elements of naturally occurring primate MAdCAM and $\alpha4\beta7$ integrin binding function, and the description and exemplification of fusion proteins containing $\alpha4\beta7$ integrin binding fragments of naturally occurring primate MAdCAM. This disclosure is adequate to show possession of the claimed fusion proteins.

The application discloses and exemplifies two species of naturally occurring human MAdCAM proteins (SEQ ID NOS:2 and 4), naturally occurring macaque MAdCAM protein (SEQ ID NO:6), and nucleic acids encoding these proteins (SEQ ID NOS:1, 3 and 5). The application also discloses methods suitable for assessing adhesion to $\alpha4\beta7$, and describes the broader class of naturally-occurring primate MAdCAM proteins by describing a combination of functional and structural features which are sufficient to distinguish the members of the genus from other materials. Specification at 13, line 3 *et seq.*, and 17, line 30 *et seq.* For example, the specification teaches:

> In a preferred embodiment, a primate MAdCAM or variant has an amino acid sequence which is at least about 55% similar, more preferably at least aobut 75% similar, and still more preferably at least about 90% similar, to a protein shown in Figure 1 (SEQ ID NO:2), Figure 2 (SEQ ID NO:43) or Figure 3 (SEQ ID NO:6).

Id. at 17, lines 23-29.

Evidence that this description, and the claim language, is sufficient to distinguish the claimed nucleic acids from other materials, such as nucleic acids encoding murine MAdCAM, is provided in the specification.

> Nucleotide alignments revealed 81.9% sequence similarity between mouse and rat MAdCAM-1 cDNAs, 41.8% similarity between mouse and macaque cDNAs, 42.1% similarity between murine and human (Clone 4) MAdCAM-1 cDNAs, and 41.8% similarity between murine and human (Clone 20) MAdCAM-1 cDNAs. ...
>
> The amino acid sequence similarities were determined to be 78.5% between mouse and rat MAdCAM-1, 44.3% between mouse and macaque, and 39% between murine and MAdCAM-1 encoded by human Clone 4.

Id. at 57, line 34 through 58, line 12.

The application contains a detailed description of the structure of naturally occurring primate MAdCAM and the relationship between structure and α4β7 integrin binding function, that is sufficient to demonstrate that Appellant was in possession of the claimed subject matter at the time the application was filed. Id. at 17, line 30 through 22, line 24. For example, the application contains a detailed description of the domain structure of murine, human and macaque MAdCAM proteins, and discloses that the amino-terminal immunoglobulin-like domains of the murine protein are involved in α4β7 integrin binding. Id. at 19, lines 11-18. The specification further teaches that naturally occurring primate MAdCAM, like the mouse protein, also comprises amino-terminal immunoglobulin-like domains and that these domains are likely to be involved in α4β7 integrin binding. Id. at 18, line 22 through page 20, line 20.

The application teaches that a conserved "GLDTSL motif" is found in naturally occurring primate MAdCAM, murine MAdCAM-1 and other Ig-like adhesion receptors. Id. at 19, lines 19-29. The conserved GLDTSL motif is taught to be located between beta sheets c and d (*i.e.*, the CD loop) of the integrin bindng domains of naturally occurring primate MAdCAM and murine MAdCAM-1, and to be required for integrin binding. Id. The specification also describes a mutation in the "GLDTSL" motif in the N-terminal domain of murine MAdCAM-1

that abolished MAdCAM-1 interaction with resting lymphocytes that expressed α4β7. Id. at 20, lines 12-19.

The specification teaches that the amino terminal region of naturally occurring primate MAdCAM contains two immunoglobulin-like domains that can be aligned with murine MAdCAM-1, and that this region of primate MAdCAM contains:

> (1) a predicted signal peptide (identical in the human proteins, and similar to the macaque and murine signal peptides); (2) two pairs of cysteine residues in the first Ig-like domain, the cysteines of each pair being separated by 3 amino acids; (3) a sequence of nine amino acids (which contains the "LDTSL" motif) in the predicted C-D loop of Ig-like domain 1, and is implicated as a general integrin recognition site (identical in each primate clone); and (4) an uncharacteristically large second immunoglobulin-like domain. ... Within this domain is an extended C'-E loop containing an abundance of negatively charged residues, which is common to each primate, murine and human MAdCAM-1 clone characterized, but which is not seen in related adhesion receptors.

Id. at 20, line 26 through 21, line 12.

The application further teaches that the mucin domain is not involved in binding to α4β7 integrin. In particular, the application discloses that some or all of the sequences in the mucin domain of naturally occurring primate MAdCAM can be deleted without abrogating integrin binding. Id. at 21, line 31 through 22, line 9.

The application also exemplifies a fusion protein that comprises the extracellular domain of naturally occurring human MAdCAM, a fusion protein that comprises the two N-terminal Ig domains of human MAdCAM, and the results of a study showing that these fusion proteins bind to cells that express α4β7 integrin. Id. at 70, line 24 through page 76, and Figure 17A-17E.

This disclosure informs the person of ordinary skill in the art of structure that is common to naturally occurring primate MAdCAM proteins (*e.g.*, the (G)LDTSL motif), and the relationship between the structure of the naturally occurring primate MAdCAM proteins and α4β7 integrin binding fragments recited in the claims and α4β7 integrin binding function. For example, based on this disclosure, and the disclosed abrogation of the α4β7 binding activity of murine MAdCAM by the introduction of a mutation in the conserved (G)LDTSL motif, a person skilled in the art would immediately recognize that mutations or truncations that alter the

(G)LDTSL motif of naturally occurring primate MAdCAM should be avoided in order to preserve $\alpha4\beta7$ binding function.

The extensive and detailed description of the application, which sets forth the complete amino acid sequence of three species of naturally occurring primate MAdCAM, the conserved (G)LDTSL motif that is a common partial structure of naturally occurring primate MAdCAM proteins, the functional characteristic of $\alpha4\beta7$ integrin binding, and a correlation between structure and $\alpha4\beta7$ integrin binding function, are adequate to convey to the person of skill in the art that the Appellant was in possession of the claimed fusion proteins at the time the application was filed.

b. The facts and circumstances of this application are distinguishable over those in the case law cited in support of the rejection, and the cited opinions are not controlling.

The record reflects that the rejection is considered to be supported by Fiers v. Revel, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993), Amgen, Inc. v. Chugai Pharmaceutical Co. Ltd., 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991), University of California v. Eli Lilly and Co., 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997) and In re Wallach, 378 F.3d 1330, 71 USPQ2d 1939 (Fed. Cir. 2004).

The reliance on Amgen, Fiers, Lilly and Wallach is misplaced because the facts and circumstances of this application are distinguishable from those in the cited opinions, and because of the limited precedential value of prior decisions in such circumstances. Accordingly, Amgen, Fiers, Lilly and Wallach do not control the issue of sufficiency of written description in this application. Amgen relates to conception of a claimed DNA. Conception is not an issue in this application, and Amgen is discussed here only to the extent that it provided concepts that have been incorporated into the law of written description.

In Amgen, the court held that conception of a claimed gene requires that the inventor "be able to define it so as to distinguish it from other materials, and to describe how to obtain it." Amgen, 927 F.2d at 1206, 18 USPQ2d at 1021. The court stated that it is not sufficient to define a DNA solely by its principal biological property (*i.e.*, the protein it encodes), but that conception occurs when "one has a mental picture of the structure of the chemical, or is able to

define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it." Id. Thus, Amgen specifically provides that conception of a claimed nucleic acid is not dependent on the inventor's ability to define the nucleic acid by its nucleotide sequence.

Similarly, under Amgen, conception of a claimed protein or fusion protein requires that the inventor be able to define the claimed protein or fusion protein so as to distinguish it from other materials and to describe how to obtain it. Conception of a claimed protein or fusion protein is not dependent on the inventor's ability to define it by its amino acid sequence.

i. The application contain a more extensive written description of the claimed invention than the application held to satisfy the written description requirement in Fiers.

In Fiers, the court adopted the conception standard of Amgen for evaluating the sufficiency of written description stating, "[i]f a conception of a DNA requires a precise definition, such as by structure, formula, chemical name, or physical properties, as we have held, then a description also requires that degree of specificity." Fiers, at 984 F.2d at 1171, 25 USPQ2d at 1606.

The Fiers case relates to an interference that involved a single count:

> A DNA which consists essentially of a DNA which codes for a human fibroblast interferon-beta polypeptide.

Id. at 1166, 25 USPQ2d at 1603.

The court held that junior party Fiers was unable to establish a date of conception prior to the filing date of his application and did not address the written description requirement of 35 U.S.C. § 112. Id. at 1169, 25 USPQ2d at 1605.

Junior party Revel attempted to establish priority based upon the filing date of his Israeli priority application, which disclosed a method for isolating a fragment of the DNA and a method for isolating a messenger RNA coding for Interferon-beta, but did not disclose a complete DNA sequence coding for interferon-beta. Id. at 1170, 25 USPQ2d at 1603 (emphasis added). In evaluating Revel's priority claim, the court focused on whether the Israeli application contained a

written description of the DNA of the count. Id. The court found that the Israeli application did not describe the DNA itself, and noted that the application did not even demonstrate that the disclosed methods would lead to the DNA. Id., 25 USPQ2d at 1605. The court held that Revel's Israeli application did contain adequate written description of the subject matter of the count. Id. at 1171, 25 USPQ2d at 1606.

In contrast, senior party Sugano's Japanese priority application disclosed the complete nucleotide sequence of a DNA coding for interferon-beta and a method for isolating that DNA. Id. The court concluded that "Sugano's application satisfies the written description requirement since it sets forth the complete and correct nucleotide sequence of a DNA coding for β-IF and thus 'convey[s] with reasonable clarity to those skilled in the art that, as of the filing date sought, [Sugano] was in possession of the [DNA coding for β-IF].'" Id. at 1172, 25 USPQ2d at 1607. Accordingly, the court affirmed the award of priority to Sugano. Id.

The subject application, unlike Revel's priority application in Fiers, discloses three species of naturally occurring primate MAdCAM by amino acid sequence, the domain structure of naturally occurring primate MAdCAM, structure that is common to naturally occurring primate MAdCAM proteins, a correlation between structure and α4β7 integrin binding function, and exemplifies two species of fusion proteins that comprise α4β7 integrin binding fragments of naturally occurring primate MAdCAM. Specification at 17, line 30 *et seq.*, 70, line 24 *et seq.*, and Figure 17A-17E. Therefore, the disclosure of the subject application is similar to, but much more extensive than that of Sugano's Japanese priority document, which the Federal Circuit held to satisfy the written description requirement of 35 U.S.C. § 112.

ii. The rationale in Lilly does not support the rejection.

In Lilly the court found claims of U.S. Patent No. 4,652,525 (the '525 patent), drawn to DNAs encoding vertebrate, mammalian or human insulin to be invalid for lack of an adequate written description of the claimed subject matter. Lilly 119 F.3d at 1567-68, 43 USPQ2d at 1404-05. The disclosure of the '525 patent includes the nucleotide sequence of a cDNA encoding rat insulin, but the patent does not include a description of the characteristics of any cDNAs encoding other vertebrate or mammalian insulins. Id. at 1567, 43 USPQ2d at 1404-05.

The court held that a description of rat insulin cDNA is not a description of the claimed broader class of vertebrate, mammalian or human cDNA, stating:

> A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus.

Id. at 1569, 43 USPQ2d at 1406.

Unlike the '525 patent in Lilly, the subject application discloses three species of naturally occurring primate MAdCAM by amino acid sequence (SEQ ID NOS:2, 4 and 6). As described in detail above, the specification also describes the domain structure of naturally occurring primate MAdCAM, structure that is common to naturally occurring primate MAdCAM proteins, a correlation between structure and $\alpha 4\beta 7$ integrin binding function, and exemplifies two species of fusion proteins that comprise $\alpha 4\beta 7$ integrin binding fragments of naturally occurring primate MAdCAM. Specification at 17, line 30 *et seq*., 70, line 24 *et seq*., and Figure 17A-17E. The application also describes methods suitable for assessing adhesion to $\alpha 4\beta 7$. Id. at 42, line 5 *et seq*., and 54, line 15 *et seq.*

The extensive disclosure and exemplification of the subject application eclipses the disclosure of the '525 patent in Lilly. In view of the extensive disclosure and exemplification of the subject application, Lilly does not support the rejection.

iii. The rationale in Wallach does not support the rejection.

Wallach was first cited by the Examiner in support of the rejection in the most recent Office Action. Office Action at 5. Accordingly, Appellant addresses Wallach for the first time in this brief.

The Examiner states that in Wallach the Federal Circuit held that "written description for a nucleic acid sequence encoding a protein required a complete intact nucleic acid sequence encoding said protein or a complete intact amino acid sequence of a protein (from which the nucleic acid sequence could be derived)." Office Action at 5. The Examiner further states that the claims "encompass amino acids for which no complete amino acid sequence has been

furnished." Id. However, the Wallach court did not create such a rule, and such a rule would be contrary to the precedent of Lilly.

In Wallach, the court affirmed the decision of the Board of Patent Appeals and Interferences affirming the rejection of claims of U.S. Patent Application No. 08/485,129 (the '129 application) as lacking written description. Wallach, 378 F.3d at 1335, 71 USPQ2d at 1944. The appealed claim was drawn to an isolated DNA that encoded Tumor Necrosis Factor Binding Protein II (TBP-II), and recited a ten amino acid partial structure of TBP-II and other biochemical characteristics of the protein.[1] Id. at 1332, 71 USPQ2d at 1941. Faced with a specification that did not disclose any nucleotide sequences that encode TBP-II, Wallach argued that the complete amino acid sequence of a protein is an inherent property of the isolated protein that was characterized by partial amino acid sequence and other biochemical characteristics, and that the complete amino acid sequence of a protein puts one in possession of all DNA sequences encoding that protein. Id. at 1332-33, 71 USPQ2d at 1941-42.

The court noted that Wallach may have been in possession of the entire genus of DNA sequences that encode the disclosed partial amino acid sequence of TBP-II, because a complete amino acid sequence may place one in possession of the genus of DNA encoding that amino acid sequence. Id. at 1333, 71 USPQ2d at 1942. However, the court affirmed the Board, concluding that the claim lacked adequate written description because the application failed to disclose any species of DNA that might support the claimed genus, and there was no evidence of any known or disclosed correlation between the combined partial structure and biochemical characteristics of TBP-II and the structure of a DNA encoding the protein. Id. at 1335, 71 USPQ2d at 1943.

Unlike the '129 application in Wallach, which did not disclose the full amino acid sequence of TBP-II or a single species of the claimed genus of DNAs, the subject application contains extensive disclosure and exemplification of the claimed subject matter. As described in detail above, the application discloses three species of naturally occurring primate MAdCAM by amino acid sequence, the domain structure of naturally occurring primate MAdCAM, structure that is common to naturally occurring primate MAdCAM proteins, a correlation between

---

[1] The court treated all claims as standing or falling with claim 11, and confined its analysis and remarks to claim 11. Wallach at 1332, 71 USPQ2d at 1941.

structure and $\alpha4\beta7$ integrin binding function, and exemplifies two species of fusion proteins that comprise $\alpha4\beta7$ integrin binding fragments of naturally occurring primate MAdCAM. Specification at 17, line 30 *et seq.*, 70, line 24 *et seq.*, and Figure 17A-17E. Again, this disclosure eclipses the disclosure of the application in Wallach.. In light of this extensive disclosure and exemplification, the facts of this application distinguish it from the application in Wallach, and the rationale for rejection in Wallach does not support the present rejection.

c. Example 14 of the Application of Guidelines demonstrates that the subject matter of claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 and 152-160 is supported by adequate written description.

The Application of Guidelines contains Example 14, which illustrate the PTO's understanding of the appropriate analysis for determining whether patent applications contain adequate written description of the subject matter of claims that recite percent amino acid sequence identity. Application of Guidelines at 53. Again, the U.S. Court of Appeals for the Federal Circuit has adopted the PTO standard, articulated in the Guidelines, as persuasive authority for determining compliance with the written description requirement for an invention that is described functionally. Enzo 269 F.3d at 1324, 63 USPQ2d at 1613.

In Example 14, the specification is said to disclose a single protein that catalyzes the reaction A → B, which has the amino acid sequence of SEQ ID NO:3. Application of Guidelines at 53. The specification contemplates, but does not exemplify, variants having all or any of the following: substitutions, insertions and deletions. Id. The specification is further said to indicate that procedures for producing such variants are conventional in the art and to disclose an assay for detecting the catalytic activity of the protein. Id. The application is said to contain the following claim:

> A protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A → B.

Id.

The analysis presented in Example 14 states that the claim has two generic embodiments (*i.e.*, (1) a protein which comprises SEQ ID NO:3; and (2) variants of SEQ ID NO:3), and focuses on whether the specification satisfies the written description requirement by describing a representative number of species within the genus. Id. at 54.

According to the analysis, SEQ ID NO:3 is novel and nonobvious, and was actually reduced to practice. Id. In addition, the specification and claim are said to reveal that:

1) the genus of proteins that are variants of SEQ ID NO:3 does not have substantial variation because all variants must have at least 95% identity to SEQ ID NO:3 and must have the specified activity; and
2) the single disclosed species (SEQ ID NO:3) is representative of the claimed genus because all members of the genus have at least 95% identity to SEQ ID NO:3 and because an assay suitable for identifying all variants that have the specified activity is disclosed. Id.

Based on these findings, the specification in Example 14 is said to meet the written description requirement of 35 U.S.C. § 112 for the claim. Id. at 55.

i. Claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 are supported by adequate written description.

Claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 are similar to Example 14 of the Application of Guidelines, because these claims define the naturally-occurring primate MAdCAM, naturally occurring human MAdCAM and $\alpha4\beta7$ integrin binding fragments of these proteins by structural features (*i.e.*, at least about 75% amino acid sequence similarity to SEQ ID NO:2, 4 or 6) and function (*i.e.*, $\alpha4\beta7$ integrin binding). However, the subject application contains a more extensive written description of the claimed invention than does the specification in Example 14 of the Application of Guidelines. For example, the application discloses three species of naturally occurring primate MAdCAM by amino acid sequence, the domain structure of naturally occurring primate MAdCAM, structure that is common to naturally occurring primate MAdCAM proteins, a correlation between structure and $\alpha4\beta7$ integrin binding function, and exemplifies two species of fusion proteins that comprise $\alpha4\beta7$ integrin binding fragments of naturally occurring primate MAdCAM. Specification at 17, line 30 *et seq.*, 70, line

24 *et seq.*, and Figure 17A-17E. The application also discloses methods suitable for assessing $\alpha4\beta7$ integrin binding, and describes the broader class of naturally-occurring primate MAdCAM proteins by describing a combination of functional and structural features which are sufficient to distinguish the members of the genus from other materials. Id. at 13, line 3 *et seq.*; and 17, line 30 *et seq.*

Applying the analysis from Example 14 of the Application of Guidelines to the claims of the subject application that recite at least about 75% amino acid sequence similarity reveals:

1) the genus of fusion proteins does not have substantial variation because the recited naturally occurring primate or naturally occurring human MAdCAM has at least about 75% amino acid sequence similarity to SEQ ID NO:2, 4 or 6 and the specified $\alpha4\beta7$ integrin binding function; and

2) the three disclosed species of naturally occurring primate MAdCAM (SEQ ID NOS:2, 4 and 6) and the two disclosed and exemplified species of $\alpha4\beta7$ integrin binding fragments of naturally occurring human MAdCAM (fusion proteins comprising the extracellular domain or the two N-terminal immunoglobulin domains of naturally occurring human MAdCAM) are representative of the genus because:

   a) the members of the genus of naturally-occurring primate (or human) MAdCAM have at least about 75% amino acid sequence similarity to SEQ ID NO:2, 4 or 6;

   b) $\alpha4\beta7$ integrin binding was exemplified in the application for two species of fusion proteins that comprise $\alpha4\beta7$ binding fragments of naturally occurring human MAdCAM; and

   c) assays suitable for identifying naturally occurring primate MAdCAM proteins and fragments thereof that bind $\alpha4\beta7$ integrin are disclosed.

Therefore, like in Example 14 of the Application of Guidelines, the instant specification provides adequate written description for the subject matter of claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 and the rejection should be reversed with respect to these claims.

ii. Claims 136-150 and 152-160 are supported by adequate written description.

Similarly, the rejection of claims 136-150 and 152-160 which recite "at least about 90% amino acid sequence similarity," and are even more similar to Example 14 of the Application of Guidelines than claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125, should be reversed. Again, these claims define the naturally-occurring primate MAdCAM, naturally occurring human MAdCAM and $\alpha 4\beta 7$ integrin binding fragments of these proteins by structural features (*i.e.*, at least about 90% amino acid sequence similarity to SEQ ID NO:2, 4 or 6) and function (*i.e.*, $\alpha 4\beta 7$ integrin binding). The subject matter of claims 136-150 and 152-160 is also supported by a more extensive written description of the claimed invention than is said to be present in the specification in Example 14 of the Application of Guidelines. Specification at 17, line 30 *et seq*., 70, line 24 *et seq*., and Figure 17A-17E.

Therefore, applying the analysis from Example 14 of the Application of Guidelines to the claims of the subject application that recite at least about 90% amino acid sequence similarity reveals:

1) the genus of fusion proteins comprising a naturally occurring primate or naturally occurring human MAdCAM, or $\alpha 4\beta 7$ integrin binding fragment thereof, does not have substantial variation because the recited naturally occurring primate or naturally occurring human MAdCAM has at least about 90% amino acid sequence similarity to SEQ ID NO:2, 4 or 6 and the specified $\alpha 4\beta 7$ integrin binding function; and

2) the three disclosed species of naturally occurring primate MAdCAM (SEQ ID NOS:2, 4 and 6) and the two disclosed and exemplified species of $\alpha 4\beta 7$ integrin binding fragments of naturally occurring human MAdCAM are representative of the genus because:

   a) the members of the genus of naturally-occurring primate (or human) MAdCAM have at least about 90% amino acid sequence similarity to SEQ ID NO:2, 4 or 6;

b) $\alpha4\beta7$ integrin binding was exemplified in the application for two species of fusion proteins that comprise $\alpha4\beta7$ binding fragments of naturally occurring human MAdCAM; and

c) assays suitable for identifying naturally occurring primate MAdCAM proteins and fragments thereof that bind $\alpha4\beta7$ integrin are disclosed.

Therefore, like in Example 14 of the Application of Guidelines, the instant specification provides adequate written description for the subject matter of claims 136-150 and 152-160 and the rejection should be reversed with respect to these claims.

d. The Guidlines illustrate the analysis that should be applied in view of the particular facts and circumstances of each case and do not establish a bright-line test.

The foregoing analysis in conjunction with Example 14 of the Application of Guidelines has been deemed unpersuasive and the rejection has been maintained, because the Examiner believes that Example 14 is not germane to the claims under consideration because the rejected claims do not recite 95% identity. Office Action at 4. Thus, Example 14 of the Application of Guidelines has been applied as a bright-line test to assess whether claims that recite percent amino acid sequence similarity are adequately supported by the specification.

Applying the Application of Guidelines in this way is legally improper because each case must be decided on its own facts. Vas-Cath 93 F.2d at 1562, 19 USPQ2d at 116. The Application of Guidelines does not establish a bright-line test, but illustrates the analysis that should be applied, in view of the particular facts and circumstances of each case, when determining whether the claimed invention is adequately described. Enzo 296 F.3d at 1324, 63 USPQ2d at 1613.

Although neither claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125, nor claims 136-150 and 152-160, recite "95% identity," they are similar to the claim in Example 14 of the Application of Guidelines because they define the naturally-occurring primate MAdCAM, naturally occurring human MAdCAM and $\alpha4\beta7$ integrin binding fragments of these proteins by structural features (*i.e.*, at least about 75% or 90% amino acid sequence similarity to SEQ ID NO:2, 4 or 6) and function (*i.e.*, $\alpha4\beta7$ integrin binding). Moreover, this application

contains a more extensive written description of the claimed invention than the specification in Example 14 of the Application of Guidelines that is sufficient to demonstrate that Applicant was in possession of the subject matter of claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125, and of the subject matter of claims 136-150 and 152-160, at the time the application was filed.

In view of this disclosure and exemplification, the person of ordinary skill in the art would immediately be able to envision a large number of fusion proteins that fall within the claims, and would reasonably conclude that Appellant was in possession of the claimed subject matter.

The written description requirement is satisfied when the specification describes the claimed invention in sufficient detail so that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. Vas-Cath 935 F.2d at 1563, 19 USPQ2d 1116. Applying the analysis illustrated in Example 14 of the Application of Guidelines to the particular facts and circumstances of this application compels a finding that the written description requirement for the subject matter of claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125, and of the subject matter of claims 136-150 and 152-160, is satisfied because one skilled in the art would reasonably conclude that Appellant had possession of the claimed invention at the time the application was filed.

## B. CLAIMS 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 AND 152-160 ARE NOT PROPERLY REJECTED UNDER 35 U.S.C. § 112, FIRST PARAGRAPH, AS CONTAINING SUBJECT MATTER THAT WAS NOT DESCRIBED IN THE SPECIFICATION IS SUCH AS WAY AS TO ENABLE ONE SKILLED IN THE ART TO MAKE OR USE THE INVENTION.

The rejection of record reflects the Examiner's opinion that the claimed subject matter is not enabled. This opinion is based on the Examiner's concern that the claimed fusion proteins can have some limited degree of amino acid sequence variation relative to SEQ ID NOS:2, 4 or 6. Office Action at 6. The Examiner states that "there is no disclosure in the specification as to what amino acid residues are important for $\alpha 4\beta 7$ integrin-binding," and that "even a single

amino acid change or mutation can destroy the function of the biomolecule in many instances." Id. The examiner concludes that which amino acids would or would not have an effect on function would be unpredictable, and that it would require undue experimentation to practice the invention. Id.

A Declaration of the Appellant under 37 C.F.R. § 1.132 providing evidence of enablement was filed in this application. ("Briskin Declaration" copy provided in Evidence Appendix.) The Examiner stated that the declaration discloses the results of studies performed using techniques that are not disclosed in the specification, and he interpreted the declaration as demonstrating that the effect of amino acid substitutions on $\alpha4\beta7$ integrin-binding could not be predicted. Id. at 6-7.

1. The rejection of claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124, 125, 136-150 and 152-160 should be reversed because any person skilled in the art could make and use the claimed fusion proteins using his knowledge of the art and the disclosure of the application.

"Enablement is not precluded by the necessity for some experimentation such as routine screening." In re Wands, 858 F.2d 731, 736-37, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). "[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." Id. at 737, 8 USPQ2d at 1404. Enablement does not require absolute predictability, but that the person of ordinary skill in the art be able to practice the invention without undue experimentation. Id. Factors to be considered in determining whether undue experimentation would be required to practice an invention included (1) the nature of the claimed invention, (2) the breadth of the claims, (3) the relative skill in the art, (4) the state of the prior art, (5) the presence or absence of working examples, (6) the quantity of experimentation necessary to make or use the invention, (7) the amount of direction or guidance presented in the application, and (8) the predictability or unpredictability of the art. Id. No one factor is determinative, and the enablement requirement is met if a preponderance of the evidence indicates that it is more likely than not that any person skilled in the art at the time the

application was filed could have produced the claimed fusion proteins without undue experimentation.

Applying this analysis, the Wands court concluded that the substantial amount of experimentation that was required to obtain high affinity IgM antibodies was not undue experimentation. Id. at 740, 8 USPQ2d at 1405. In Wands, claims to a method of using high affinity IgM antibodies were rejected as lacking enablement, and the rejection was affirmed by the Board. Id. at 734, 8 USPQ2d at 1402. During prosecution, Wands submitted a declaration which provided details of the experimentation required to produce an antibody as recited in the rejected claims. Id. at 738, 8 USPQ2d at 1405. The declaration disclosed that the first four attempts to make hybridomas that produce such an antibody failed, and resulted in no hybridomas. Id. The next six attempts did result in hybridomas that produced antibodies of the required specificity. Id. From these hybridomas, 143 hybridomas that produced "high-binding" antibodies were obtained. Id. A few of the hybridomas that produced "high-binding" antibodies were chosen for further study, and the remainder of the hybridomas were frozen and not subjected to any further study. Id. The court noted that the declaration showed that only 4 out of 143 hybridomas, or 2.8 percent, were proved to fall within the claims, and the antibodies proved to fall within the claims were isolated from only 2 out of 10 fusion experiments that were performed. Id. at 739, 8 USPQ2d at 1405.

The court determined that this quantity of experimentation was not undue because Wands' disclosure provided direction and guidance on how to practice the invention and disclosed working examples of the invention, there was a high level of skill in the art, and all methods needed to practice the invention were well known. Id. at 740, 8 USPQ2d at 1406-07. The court noted that the art involved preparing hybridomas and screening them to identify those that produce an antibody with desired characteristics, and that practitioners of the art were prepared to screen negative hybridomas in order to find one that makes a desired antibody. Id. The court concluded that this type of activity was routine in the art and did not constitute undue experimentation. Id.

Applying the factors enumerated in Wands for determining whether undue experimentation would be required to make and use the claimed fusion proteins demonstrates that the claims are enabled.

a. Claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 are enabled.

The enablement factors enumerated in Wands demonstrates that the claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 are enabled.

i. Factors 1 and 2: The nature of the claimed invention and breadth of the claims.

The claims are drawn to fusion proteins or hybrid immunoglobulins. The claimed fusion proteins or hybrid immunoglobulins comprise a naturally occurring primate MAdCAM or an $\alpha4\beta7$ integrin-binding fragment thereof, a naturally occurring human MAdCAM or an $\alpha4\beta7$ integrin-binding fragment thereof, a primate MAdCAM moiety, or a primate MAdCAM or $\alpha4\beta7$ integrin-binding portion thereof. The claims recite that the naturally occurring primate or human MAdCAM, primate MAdCAM moiety or primate MAdCAM has a stated amount of amino acid sequence similarity (at least about 75% or 90%) to a recited amino acid sequence.

ii. Factors 3 and 4: The relative skill in the art and the state of the prior art.

The relative skill in the art of molecular biology at the time the application was filed was high. At that time, it was routine for a person skilled in the art to produce recombinant proteins and fusion proteins that comprised an amino acid sequence with at least about 75% amino acid sequence similarity to a reference amino acid sequence, and to assess binding function of such proteins and fusion proteins (*e.g.*, immunoglobulin superfamily proteins such as MAdCAM) using conventional methods. A variety of suitable methods for producing and evaluating the binding activity of such proteins were well-known in the art at that time. See, *e.g.*, Specification at 15, line 22 through 17, line 29.

Several immunoglobulin superfamily adhesion receptors are in the prior art, including VCAM-1, ICAM-1 and murine MAdCAM. The binding specificities of these immunoglobulin superfamily adhesion receptors were known at the time the application was filed. Specification at 3, line 4 through 5, line 4. The domain structures of these immunoglobulin superfamily

adhesion receptors and sequence motifs required for integrin-binding were also known in the art. Specification at 19, line 11 through 20, line 19.

iii. Factor 5: The presence of working examples.

The specification discloses the amino acid sequences of two species of naturally occurring human MAdCAMs (SEQ ID NOS: 1 and 3), the amino acid sequence of a naturally occurring macaque MAdCAM (SEQ ID NO:6) and exemplifies adhesion assays using cells that express the MAdCAM proteins.

The specification discloses and exemplifies two fusion proteins that each comprise an $\alpha4\beta7$ binding fragment of naturally occurring human MAdCAM, and a method for identifying fusion proteins that bind $\alpha4\beta7$ integrin. Id. at 73-76. One of the exemplified fusion proteins comprises the extracellular domain of human MAdCAM (SEQ ID NO:4) and the other comprises the two amino-terminal immunoglobulin-like domains of human MAdCAM (SEQ ID NO:4). Id. The specification also discloses that the exemplified fusion proteins stained cells that express $\alpha4\beta7$ integrin, demonstrating that they bound $\alpha4\beta7$ integrin. Id.

iv. Factors 6 and 7: The quantity of experimentation necessary to make or use the invention and the amount of direction or guidance presented in the application.

The person of ordinary skill in the art would be able to practice the claimed invention following the guidance of the specification, using no more than routine experimentation. Methods suitable for preparing variants of proteins that contain amino acid additions, deletions and/or substitutions, to produce an amino acid sequence with at least about 75% amino acid sequence similarity to a reference amino acid sequence, were well-known in the art at the time the application was filed. As discussed above, the specification includes working examples of two fusion proteins that each comprise an $\alpha4\beta7$ binding fragment of human MAdCAM, and a method for identifying fusion proteins that bind $\alpha4\beta7$ integrin. Id.

The specification contains adequate guidance to enable a person skilled in the art to make the claimed fusion proteins. The specification includes a detailed discussion of MAdCAM structure. Id. at 17, line 30 through 22, line 24. This discussion points out regions and particular

amino acid motifs of naturally occurring primate MAdCAM that are important for $\alpha 4\beta 7$ integrin binding. In particular, naturally occurring human and macaque MAdCAM are taught to have two amino-terminal immunoglobulin-like domains that are homologous to those of murine MAdCAM. Id. at 18, lines 21-26. The specification teaches that domain 1 of murine MAdCAM and of ICAM-1, ICAM-2 and ICAM-3, and domains 1 and 4 of VCAM-1 contain a short amino acid motif (G-(I/L)-(D/E)-(T/S)-(P/S)-L) that is located between β sheets c and d of the proteins (the C-D loop). Id. at 19, lines 19-29. The specification further teaches that this GLDTSL motif is also found in the primate and human MAdCAMs disclosed in the application. Id.

The specification includes a discussion of published studies that demonstrated that mutations in the GLDTSL motif in ICAM-1 or VCAM-1 dramatically affected binding to LFA-1 or $\alpha 4\beta 7$ integrin, respectively. Id. The specification further teaches that a mutation in the GLDTSL motif in murine MAdCAM abolished interaction with cells that expressed $\alpha 4\beta 7$ integrin, and that the GLDTSL motif is required for binding of murine MAdCAM to $\alpha 4\beta 7$ integrin. Id. at 20, lines 11-19. The specification also teaches that each primate clone disclosed contains "a sequence of nine amino acids (which contain the "LDTSL" motif) in the predicted C-D loop of the Ig-like domain 1, and is implicated as a general integrin recognition site ...." Id. at 20, lines 31-34.

Thus, the specification teaches the person of ordinary skill in the art that the C-D loop in immunoglobulin-like domain 1, and the GLDTSL motif or LDTSL motif in particular, are important for binding to $\alpha 4\beta 7$ integrin, and that amino acid additions, deletions and/or substitutions in the C-D loop could alter and possibly abrogate binding to $\alpha 4\beta 7$ integrin. Accordingly, the specification provides ample guidance regarding the structure-function relationship of MAdCAM to enable any person skilled in the art to make the claimed fusion proteins without undue experimentation.

v. Factor 8: The predictability or unpredictability of the art.

The Examiner considers the art to be unpredictable. However, even if this were true, the patent statutes do not require absolute predictability, only that it would not require undue experimentation to make and use the claimed invention.

In view of the foregoing, claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 are supported by an enabling disclosure because any person skilled in the art at the time the application was filed could have made and used the claimed invention by following the teachings, guidance and examples of the application and existing knowledge in the art without undue experimentation. At the time the application was filed, preparing and screening proteins, such as fusion proteins, to ascertain binding properties was routine in the art, and did not constitute undue experimentation. This type of routine screening is analogous to the screening of hybridomas to identify those hybridomas that produce a desired antibody which the Wands court determined was not undue experimentation. Wands, 858 F.2d at 740, 8 USPQ2d at 1405. (Holding that the claimed antibodies could be made without undue experimentation when antibodies produced by only 4 out of 143 hybridomas, or 2.8 percent, were proved to fall within the claims, and the antibodies proved to fall within the claims were isolated from only 2 out of 10 fusion experiments that were performed.)

b. Claims 136-150 and 152-160 are supported by an enabling disclosure.

Claims 136-150 and 152-160 are enabled for substantially the same reasons as claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125. However, claims 136-150 and 152-160 recite that the naturally occurring primate or human MAdCAM, primate MAdCAM moiety or primate MAdCAM has at least about 90% amino acid sequence similarity to a recited amino acid sequence. Accordingly, theses claims are drawn to a smaller subgenus, and less direction or guidance is needed to enable the scope of these claims than is needed to enable claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125.

Claims 136-150 and 152-160 are supported by an enabling disclosure because any person skilled in the art at the time the application was filed could have made and used the claimed invention by following the teachings, guidance and examples of the application and existing knowledge in the art without undue experimentation. At the time the application was filed, preparing and screening proteins, such as fusion proteins that comprised an amino acid sequence with at least about 90% amino acid sequence similarity to a reference amino acid sequence, to ascertain binding properties was routine in the art, and did not constitute undue experimentation.

This type of routine screening is analogous to the screening of hybridomas to identify those hybridomas that produce a desired antibody which the Wands court determined was not undue experimentation. Id.

2. The rejection should be reversed because the Briskin Declaration provides objective evidence of enablement.

The Briskin Declaration describes a study in which a number of fusion proteins comprising portions of human MAdCAM that contained single amino acid substitutions were prepared and tested for binding to $\alpha4\beta7$ integrin. The mutations were made in portions of human MAdCAM that correspond to regions of other immunoglobulin-like adhesion molecules that are important for integrin binding, namely the CD loop, EF loop, C'E loop and FG loop. Briskin Declaration at 4, lines 22-26. Of the 31 fusion proteins generated, 14 displayed mean binding to $\alpha4\beta7$ integrin at 80% to 100% of the control (binding of wild type MAdCAMIg fusion protein) in an adhesion assay. Id. at the Table. Thus, even though the study specifically introduced amino acid substitutions into regions of human MAdCAM that the specification teaches are important for binding to $\alpha4\beta7$ integrin, about half of the fusion proteins produced retained at least 80% of the $\alpha4\beta7$ binding activity.

The production of fusion proteins and screening the fusion proteins for the desired $\alpha4\beta7$ integrin binding function described in the Briskin Declaration were routine activities in the art at the time the application was filed, and are analogous to the screening of hybridomas to identify those hybridomas that produce a desired antibody, which the Wands court determined was not undue experimentation. Wands, 858 F.2d at 740, 8 USPQ2d at 1405. Under the circumstances, any experimentation required to practice the invention is not undue.

The methods employed in the studies described in the Briskin Declaration were disclosed in the application or art-known at the time the application was filed. For example, the specification teaches and exemplifies methods for producing fusion proteins comprising $\alpha4\beta7$ integrin-binding fragments of human MAdCAM and assessing $\alpha4\beta7$ binding. Specification at page 73-76. Methods that were art-known need not be disclosed in a patent application, and many suitable methods for introducing amino acid substitutions (*e.g.*, substituting a desired

amino acid residue with alanine) were well-known and conventional in the art at the time the application was filed.

C. CLAIMS 24-26, 28-31, 105-108, 111, 113, 115, 116, 118, 120, 121, 124, 126-142, 144-147, 149, 150, 152, 154, 155 AND 157-160 ARE NOT PROPERLY REJECTED UNDER 35 U.S.C. § 103(a) AS BEING OBVIOUS OVER BUTCHER *ET AL.* (WO 94/13312, REFERENCE AD OF RECORD) IN VIEW OF VONDERHEIDE *ET AL.* (U.S. PATENT NO. 5,599,676, REFERENCE AB OF RECORD) AND ERLE *ET AL.* (*J. IMMUNOL. 153*:517-528 (1994); REFERENCE AX3 OF RECORD).

According to the Examiner, Butcher *et al.* teaches fusion proteins comprising murine MAdCAM and an Ig constant region. Office Action at 7-8.

Vonderheide *et al.* is said to teach methods to isolate nucleic acids encoding molecules that bind $\alpha4\beta7$ integrin that require the use of human cell lines expressing $\alpha4\beta7$ integrin and human cells expressing MAdCAM, and to teach nucleic acids encoding molecules that bind $\alpha4\beta7$. Id.

However, Vonderheide *et al.* in fact discloses and claims a general method for isolating a cDNA that encodes an $\alpha4$ integrin receptor. There is no mention of human MAdCAM or disclosure of any $\alpha4$ integrin receptor in Vonderheide *et al.*

Erle *et al.* is said to teach that human MAdCAM binds to $\alpha4\beta7$. Erle *et al.* is also said to disclose a source of nucleic acids encoding human MAdCAM. Id. at 9. Erle *et al.* is also said to teach that many disclosed integrins recognize ligands across species, which the Examiner considers to be evidence that $\alpha4\beta7$ integrin binds human MAdCAM. Id.

Erle *et al.* does not teach that human MAdCAM binds to $\alpha4\beta7$ integrin. Erle *et al.* expressly teaches that the human homologue of MAdCAM-1 had not been identified, and consequently transfected cells that expressed murine MAdCAM-1 were used in their experiments. Reference AX3 at 525, left column.

According to the rejection of record, it would have been obvious to make the claimed invention because Butcher *et al.* teach murine MAdCAM/Ig fusion proteins and Vonderheide *et

*al.* and Erle *et al.* provide the means to produce human or primate MAdCAM protein. Office Action at 8. The Examiner states that the person of ordinary skill in the art would have been motivated to modify the teachings of the references because Butcher *et al.* teach that MAdCAM fusion proteins that bind $\alpha4\beta7$ could have been used for a variety of purposes. Id. It appears that the requisite reasonable expectation of success in arriving at the claimed invention is provided because Vonderheide *et al.* is an issued U.S. patent and the claims are presumed to be enabled. Id. at 9.

The Examiner considers the rejection to comport with In re Deuel, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995) and Ex parte Goldgaber, 41 USPQ2d 1172 (Bd. Pat. App. & Inter. 1995).

1. The rejection should be reversed because the combined teachings of the cited references do not provide the suggestion or motivation, or reasonable expectation of success, necessary to establish a *prima facie* case under 35 U.S.C. § 103.

The claimed invention is not obvious over the cited references, because none of the references either individually or in combination suggests the claimed fusion proteins. A finding that the claimed invention is obvious under 35 U.S.C. § 103 requires that (1) "the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process;" and (2) that "the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success." In re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in Appellant's disclosure. Id. When novel compounds are claimed using structural terms, a *prima facie* case of obviousness requires that the prior art suggest the claimed compounds themselves to the person of ordinary skill in the art. Deuel 51 F.3d at 1557, 34 USPQ2d at 1214. A particular result is not made obvious by a general incentive or the existence of techniques suitable to achieve the result. Id. at 1559, 34 USPQ2d at 1216.

The disclosure of Butcher *et al.* does not suggest the particular claimed fusion proteins themselves to the person of skill in the art or provide a reasonable expectation of success, because murine MAdCAM and primate MAdCAM have a very low degree of sequence similarity, and cDNAs encoding these proteins do not cross hybridize. Butcher *et al.* establishes that murine MAdCAM, nucleic acids encoding murine MAdCAM and general methods for isolating and cloning nucleic acids encoding adhesion molecules were known to exist at the time the invention was made. However, primate MAdCAM and murine MAdCAM have a very low degree of sequence similarity.[2] In fact, cDNAs encoding murine MAdCAM and primate MAdCAM have such a low degree of sequence similarity that initial attempts to isolate a cDNA encoding primate MAdCAM by low stringency cross-hybridization with a nucleic acid encoding murine MAdCAM -1 were unsuccessful. Evidence of this was provided by Appellant's submission of Shyjan A.M. *et al.* (Reference AX4 of record, copy provided in Evidence Appendix), a later article he coauthored. Shyjan A.M. *et al.* teach that "[i]nitial attempts to clone the human homologue to murine MAdCAM -1 by low stringency cross-hybridization suggested that nucleotide conservation between murine MAdCAM -1 and higher species was poor." Reference AX4 at 2853, left column.

The secondary references, Vonderheide *et al.* and Erle *et al.*, do not remedy the defect in the teachings of Butcher *et al.* As pointed out above, Vonderheide *et al.* discloses and claims a general method for isolating a cDNA that encodes an $\alpha4$ integrin receptor. There is no mention of human MAdCAM or disclosure of even one $\alpha4$ integrin receptors in Vonderheide *et al.*, and Erle *et al.* does not teach that human MAdCAM binds to $\alpha4\beta7$. Erle *et al.* expressly teach that the human homologue of MAdCAM-1 had not been identified, and consequently they used transfected cells that expressed murine MAdCAM-1 in their experiments. Reference AX3 at 525, left column. Therefore, at best, Vonderheide *et al.* discloses a method suitable for isolating a cDNA that encodes an $\alpha4$ integrin receptor, and Erle *et al.* demonstrates that primate or human MAdCAM was not known.

---

[2] The amino acid sequence similarities were determined to be 78.5% between mouse and rat MAdCAM-1, 44.3% between mouse and macaque, and 39% between murine and MAdCAM-1 encoded by human Clone 4. Specification at 58, lines 9-12.

In sum, the references cited by the Examiner teach murine MAdCAM, which has only limited sequence similarity to naturally occurring primate MAdCAM (Butcher *et al.*), a belief that human or primate MAdCAM is likely to exist and bind $\alpha4\beta7$ integrin (Erle *et al.*), and the existence of a possible method for cloning receptors for $\alpha4$ integrins (Vonderheide *et al.*).

These teachings do not establish a *prima facie* case of obviousness against the claimed fusion proteins, because the amino acid sequence disclosed by Butcher *et al.* is so dissimilar from the amino acid sequence of primate or human MAdCAM that it cannot be deemed to reasonably suggest the claimed fusion proteins. Moreover, the cited references are devoid of any nucleotide sequence data, any amino acid sequence data or any other teachings that would have reasonably suggested the particular claimed fusion proteins to the person of ordinary skill in the art. The combined teachings of the references do not create a *prima facie* case, because they do not suggest the claimed fusion proteins. Moreover, the existence of a general incentive and techniques suitable to arrive at the claimed invention is not sufficient to establish a *prima facie* case. Deuel, 51 F.3d at 1559, 34 USPQ2d at 216.

The record demonstrates that these deficiencies in the teachings of the prior art have been dismissed or not appreciated, because the Examiner states that "[t]he method taught by Vonderheide *et al.* uses $\alpha4\beta7$ binding to clone the pertinent molecule, thus overcoming any need for any amino acid data from the protein to be cloned." Office Action, at 9. Even if this statement is true, it does not cure the deficiency in the teachings of the prior art and create a *prima facie* case of obviousness. Appellant does not argue that the prior art must disclose amino acid sequence data in order to create a *prima facie* case. A prior art teaching that reasonably suggests the particular claimed fusion proteins to the person of ordinary skill in the art and provide a reasonable expectation of success is required. But the cited references do not meet this standard and merely demonstrate that primate or human MAdCAM was not known at the time the application was filed, and disclose a method that might have been used to obtain a nucleic acid encoding primate or human MAdCAM if they existed. Thus, the cited combination of references creates nothing more than a hope or, at best, a research plan that might have led the person of ordinary skill in the art to isolate the claimed nucleic acids. According to Deuel, such a hope or research plan does not reasonably suggest the claimed fusion proteins themselves,

particularly when, as here, primate and human MAdCAM have very limited sequence similarity to prior art murine MAdCAM.

a. A *prima facie* case against claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 has not been established.

A *prima facie* case has not been established against claims 24-26, 28-32, 105-108, 111-113, 115, 116, 118-121, 124 and 125 because the combined teaching of the cited references fail to suggest the amino acid sequence of a naturally occurring primate or human MAdCAM that binds $\alpha4\beta7$ integrin and has at least 75% amino acid sequence similarity to SEQ ID NO:2, 4 or 6. At best, the cited references provide a research plan that might have been used to arrive at the claimed invention.

b. A *prima facie* case against claims 136-150 and 152-160 has not been established.

A *prima facie* case has not been established against claims 136-150 and 152-160 which recite at least 90% amino acid sequence similarity to SEQ ID NO:2, 4 or 6. These claims are drawn to a smaller subgenus of fusion proteins than claims that recite at least 75% amino acid sequence similarity, and therefore a more precise motivation or suggestion must be found in the prior art to establish a *prima facie* case. However, there is nothing in the combined teachings of the cited references that suggests an amino acid sequence that meets the limitations of the claims. At best, the cited references provide a research plan that might have been used to arrive at the claimed invention.

c. A *prima facie* case against claims 126-135 has not been established.

Claims 126-135 recite that the fusion protein comprises a primate MAdCAM moiety that has a particular amino acid sequence (SEQ ID NO:2 or SEQ ID NO:4) or comprises an $\alpha4\beta7$ integrin binding portion of a polypeptide that has a particular amino acid sequence (SEQ ID NO:2 or SEQ ID NO:4) and comprises the N-terminal immunoglobulin-like domain.

The cited combination of references contains no teaching that reasonably suggests the amino acid sequence of SEQ ID NO:2, the amino acid sequence of SEQ ID NO:4, the amino acid sequence of an $\alpha 4\beta 7$ integrin binding portion of SEQ ID NO:2 which comprises the N-terminal immunoglobulin-like domain, or the amino acid sequence of an $\alpha 4\beta 7$ integrin binding portion of SEQ ID NO:4 which comprises the N-terminal immunoglobulin-like domain, to a person of ordinary skill in the art. Accordingly, a *prima facie* case has not been established.

2. The rejection should be reversed, because it is contrary to mandatory legal authority.

The rejection is inconsistent with Deuel and Goldgaber, because the disclosure in the cited references does not establish a *prima facie* case and would not have led inevitably to the claimed nucleic acids.

Deuel unambiguously articulates a fundamental rule of law for analyzing obviousness under 35 U.S.C. § 103: the existence of technology that is suitable for producing a novel composition does not on its own render that novel composition obvious. Deuel, 51 F.3d at 1559, 34 USPQ2d at 216. Therefore, Deuel controls the analysis in this application, even though the ultimate question of obviousness under § 103 is determined on the particular facts of this case. Goldgaber and Deuel agree on this point.

> We are mindful of the holding in Bell, and the recently issued opinion In re Deuel, ... reaffirming the principle that a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question of whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggests the claimed DNAs.

Goldgaber, 41 USPQ2d at 1176.

The rejection is inconsistent with Deuel and Goldgaber and should be reversed, because the cited prior art in this case contains no amino acid sequence data, no nucleotide sequence data, and no other teachings that relate to the particularly claimed fusion proteins. Thus, the person of ordinary skill in the art is further away from Appellant's claimed invention than he was in Deuel, with nothing to point him toward the particular claimed fusion proteins. Furthermore, unlike in

Goldgaber, the teachings of the cited references do not have a degree of specificity that would have "led inevitably" to the particular claimed fusion proteins.

a. The rejection is contrary to the controling precedent of Deuel.

In Deuel, the application at issue disclosed the purification and characterization of "heparin-binding growth factor" (HBGF) from bovine uterine tissue, and disclosed the amino acid sequence of the first 25 N-terminal amino acids of HBGF. Deuel, 51 F.3d at 1555, 34 USPQ2d at 1212. The application also disclosed the nucleotide sequence of a bovine and a human cDNA encoding HBGF. Id.

Claims drawn to nucleic acids encoding human heparin-binding growth factor were rejected by the Examiner under 35 U.S.C. § 103 as being obvious over a reference that disclosed the first 19 amino-terminal amino acids of a "heparin-binding brain mitogen" ("Bohlen") and a reference that described a method for isolating DNAs or cDNAs by screening a DNA or cDNA library with a gene probe ("Maniatis"). Id. at 1555-56, 34 USPQ2d at 1212-1214. The 19 amino acid sequence disclosed by Bohlen matched the first 19 amino acids of the N-terminal amino acid sequence disclosed in Deuel's application. Id. at 1556, 34 USPQ2d at 1213. The court reversed the rejection stating:

> Because Deuel claims new chemical entities in structural terms, a *prima facie* case of unpatentability requires that the teachings of the prior art suggest *the claimed compounds* to a person of ordinary skill in the art.

Id. at 1557, 34 USPQ2d at 1214. The court further stated:

> A general incentive does not make obvious a particular result, nor does the existence of techniques by which those efforts can be carried out. Thus, Maniatis's teachings, even in combination with Bohlen, fail to suggest the claimed invention.

Id. at 1559, 34 USPQ2d at 1216.

Like in Deuel, this application claims new chemical entities (*i.e.*, fusion proteins) in structural terms, and the cited references are devoid of any teachings relating to the particular claimed fusion proteins. Deuel makes clear that incentive does not make obvious a particular

result, nor does the existence of techniques by which efforts to achieve the incentive can be carried out. Id.

The facts of this application militate against a finding of obviousness more strongly than the facts in Deuel. In Deuel, the cited prior art disclosed a 19 amino acid sequence that matched the first 19 amino acids of the N-terminal amino acid sequence disclosed in Deuel's application. Id. at 1556, 34 USPQ2d at 1213. In contrast, the prior art cited in this application contains no amino acid sequence data, no nucleotide sequence data, and no other teachings that relate to the particularly claimed fusion proteins. The absence of amino acid sequence or nucleotide sequence data from the prior art is evidence of nonobviousness, because it places the person of skill in the art further away from the claimed invention than he was in Deuel, with nothing to point him toward the particular claimed fusion proteins.

b. The facts and circumstances of the application are distinguishable from Goldgaber.

In Goldgaber, the Board distinguished Deuel and held that claims drawn to a nucleic acid which hybridizes to message for beta-amyloid polypeptide of Alzheimer's disease and which hybridizes with an oligonucleotide probe having a nucleotide sequence disclosed in Figure 1 of the application, were rejected as obvious over Glenner *et al.* (U.S. Patent No. 4,666,829; "Glenner") and Huynh *et al.* ("Huynh"). Goldgaber 41 USPQ2d at 1173. The Board, clearly mindful of Federal Circuit precedent, correctly set a high standard for distinguishing Bell and Deuel that focuses on the specific teachings of the cited references and the results that those teachings would inevitably produce. Id. at 1174-1177 (emphasis added).

In Goldgaber, the primary reference, Glenner, disclosed the amino acid sequence of Alzheimer's Amyloid Polypeptide (AAP), the sequences of two sets of oligonucleotide probes suitable for isolating a gene that encodes AAP, methods for performing hybridization reactions, and use of the oligonucleotides in diagnostic assays. Id. at 1173. The secondary reference Huynh disclosed methods for constructing and screening cDNA libraries. Id. Figure 1 of Goldgaber's application included an illustration of the nucleotide sequence encoding beta-amyloid polypeptide and the amino acid sequence of the beta-amyloid polypeptide. Id. The Board found that Glenner disclosed "clearly and unequivocally that it *is* possible to ascertain the

base sequence of the gene coding for AAP, ... [and] the meaning [sic] for accomplishing that result, *i.e.*, two sets of fully degenerate probes." Id. at 1176. The Board also found that Glenner put the person of skill in the art in possession of the probes which it characterized as being the key to success. Id., at 1174. The Board found that the combined teachings of Glenner and Huynh "would have led inevitably to a clone of DNA meeting the limitations recited in claim 4," and stated that "Glenner puts the key in the lock of the door of success." Id. at 1175 (emphasis added).

The Board also found factual distinctions between the teachings of Glenner and Huynh and the teachings of the references in Bell and Deuel.

> Conspicuous by its absence from Rinderknecht or Bohlen [the primary references in Bell and Deuel, respectively] is any teaching relating to the DNA cDNA or the gene coding for the polypeptide of interest. Not only is the "primary" reference Glenner more comprehensive than the primary references in Bell or Deuel, but the "secondary" reference Huynh is also stronger than the secondary references in those cases.

Id. at 1176.

The Board affirmed the rejection under 35 U.S.C. § 103 and distinguished Deuel stating:

> The facts before us, however, present a different issue [than was presented in Deuel] and a more compelling case of obviousness because Glenner discloses more than the amino acid sequence of AAP. Glenner constructs a "bridge" of information leading from the polypeptide AAP via the oligonucleotides corresponding to its amino acid sequence to the gene coding for AAP.
>
> Glenner puts a person having ordinary skill in possession of two sets of fully degenerate probes, and Huynh discloses specific information pertaining to the construction and screening of a suitable cDNA library. The information in the Glenner patent, when combined with the Huynh reference, provide a reasonable expectation of success which is all that is required for obviousness under 35 USC 103.

Id. at 1177 (emphasis added).

Thus, the Board distinguished Deuel based upon the disclosure of oligonucleotide probes in Glenner, which provided a "bridge" of information that would have "led inevitably" to the claimed nucleic acid, and articulated a high standard for distinguishing Bell and Deuel that

focuses on the specific teachings of the cited references, and the results that those teachings would inevitably have produced. Id. at 1174, 1177.

This application is unlike Goldgaber, because it does not present facts that can be distinguished from Deuel. Critical to the Board's decision in Goldgaber was the disclosure in Glenner of the nucleotide sequences of two sets of oligonucleotide probes targeted to areas of low degeneracy in the AAP, methods for performing hybridization reactions, and use of the oligonucleotides in diagnostic assays. Id. The Board viewed these teachings as forming a "bridge" of information that "led inevitably" to the claimed nucleic acid. Id. There is no such bridge in this case.

Vonderheide *et al.* does not provide the "bridge" even though it is an issued U.S. Patent, because it lacks the specificity required to provide a reasonable expectation that the disclosed and claimed method, combined with the teachings of Butcher *et al.* and Erle *et al.*, would have "led inevitably" to the claimed fusion proteins. The method of Vonderheide *et al.* is a general method which is taught to be suitable for isolating a cDNA that encodes any receptor that binds $\alpha4$ integrins, which include but are not limited to $\alpha4\beta1$, $\alpha4\beta7$ and $\alpha4$ itself. Reference AB at column 4, lines 27-32. These general teachings are very different from the disclosure of specific oligonucleotide probes targeted to areas of low degeneracy in Goldgaber.

D. CLAIMS 32, 112, 119, 125, 143, 148, 153 AND 156 ARE NOT PROPERLY REJECTED UNDER 35 U.S.C. § 103(a) AS BEING OBVIOUS OVER BUTCHER *ET AL.* (WO 94/13312, REFERENCE AP OF RECORD) IN VIEW OF VONDERHEIDE *ET AL.* (U.S. PATENT NO. 5,599,676, REFERENCE AB OF RECORD) AND ERLE *ET AL.* (*J. IMMUNOL. 153*:517-528 (1994); REFERENCE AX3 OF RECORD), AND FURTHER IN VIEW OF CAPON *ET AL.* (U.S. PATENT NO. 5,656,335; REFERENCE AF OF RECORD).

1. A *prima facie* case against claims 32, 112, 119, and 125 has not been established.

The claims are not obvious and the rejection should be reversed for the reasons discussed herein with respect to the "Ground of Rejection C" rejection of claims 24-26, 28-31, 105-108, 111, 113, 115, 116, 118, 120, 121 and 124. Capon *et al.* adds nothing to the rejection because Capon *et al.* contains no teachings that relate to primate or human MAdCAM or that suggest the claimed fusion proteins.

Accordingly, the combined teachings of the references fail to suggest the claimed fusion proteins.

2. A *prima facie* case against claims 143, 148, 153 and 156 has not been established.

The claims are not obvious and the rejection should be reversed for the reasons discussed herein with respect to the "Ground of Rejection C" rejection of claims 136-142, 144-147, 149-150, 152, 154, 155 and 157-160. Capon *et al.* adds nothing to the rejection because Capon *et al.* contains no teachings that relate to primate or human MAdCAM or that suggest the claimed fusion proteins. Accordingly, the combined teachings of the references fail to suggest the claimed fusion proteins.

In view of the foregoing arguments and legal authority, reversal of the rejections is requested.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Robert H. Underwood

Robert H. Underwood
Registration No. 45,170
Telephone: (978) 341-0036
Facsimile: (978) 341-0136

Concord, MA 01742-9133
Date: August 14, 2006

CLAIMS APPENDIX

24. A fusion protein comprising a naturally occurring primate MAdCAM, wherein said naturally occurring primate MAdCAM binds $\alpha 4\beta 7$ integrin and has at least about 75% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

25. The fusion protein of Claim 24, comprising a first moiety and a second moiety, wherein said first moiety is a naturally occurring primate MAdCAM and said second moiety is at least a portion of an immunoglobulin chain.

26. The fusion protein of Claim 25, wherein said first moiety is joined at its C-terminal end to the N-terminal end of the second moiety.

28. The fusion protein of Claim 25, wherein the second moiety is at least a portion of an immunoglobulin heavy chain constant region.

29. The fusion protein of Claim 28, wherein the immunoglobulin heavy chain is of the IgG class.

30. The fusion protein of Claim 28, wherein the second moiety comprises hinge, CH2 and CH3 domains of an immunoglobulin heavy chain.

31. A hybrid immunoglobulin comprising a fusion protein of Claim 25.

32. The hybrid immunoglobulin of Claim 31, wherein said hybrid immunoglobulin is a homodimer.

105. The fusion protein of Claim 24 wherein said primate MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or a nucleic acid that shares at least about 75% nucleotide sequence similarity with SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

106. The fusion protein of Claim 24 wherein said primate MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or a nucleic acid that shares at least about 90% nucleotide sequence similarity with SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

107. A fusion protein comprising an $\alpha4\beta7$ integrin-binding fragment of a naturally occurring primate MAdCAM, wherein said primate MAdCAM has at least about 75% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6, and said $\alpha4\beta7$ integrin-binding fragment comprises the N-terminal immunoglobulin-like domain of said primate MAdCAM.

108. The fusion protein of Claim 107 wherein said $\alpha4\beta7$ integrin-binding fragment is selected from the group consisting of a fragment comprising the entire extracellular domain of

primate MAdCAM and a fragment comprising the two N-terminal immunoglobulin domains of primate MAdCAM.

111. A hybrid immunoglobulin comprising a fusion protein of Claim 107.

112. The hybrid immunoglobulin of Claim 111, wherein said hybrid immunoglobulin is a homodimer.

113. A fusion protein comprising a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds $\alpha4\beta7$ integrin and has at least about 75% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4.

115. The fusion protein of Claim 113 wherein said human MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or a nucleic acid that shares at least about 75% nucleotide sequence similarity with SEQ ID NO:1 or SEQ ID NO:3.

116. The fusion protein of Claim 113 wherein said human MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or a nucleic acid that shares at least about 90% nucleotide sequence similarity with SEQ ID NO:1 or SEQ ID NO:3.

118. A hybrid immunoglobulin comprising a fusion protein of Claim 113.

119. The hybrid immunoglobulin of Claim 118, wherein said hybrid immunoglobulin is a homodimer.

120. A fusion protein comprising an α4β7 integrin-binding fragment of a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds α4β7 integrin and has at least about 75% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4, and said α4β7 integrin-binding fragment comprises the two N-terminal immunoglobulin-like domains of said human MAdCAM.

121. The fusion protein of Claim 120, wherein said α4β7 integrin-binding fragment is selected from the group consisting of a fragment comprising the entire extracellular domain of human MAdCAM and a fragment comprising the two N-terminal immunoglobulin domains of human MAdCAM.

124. A hybrid immunoglobulin comprising a fusion protein of Claim 120.

125. The hybrid immunoglobulin of Claim 124, wherein said hybrid immunoglobulin is a homodimer.

126. A fusion protein comprising a primate MAdCAM moiety, wherein said primate MAdCAM moiety has binding affinity for α4β7 integrin and comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2 and the amino acid

sequence of an $\alpha 4\beta 7$ integrin-binding portion of the polypeptide shown in Figure 1 (SEQ ID NO:2), wherein said $\alpha 4\beta 7$ integrin-binding portion comprises the N-terminal immunoglobulin-like domain.

127. The fusion protein of Claim 126 wherein said $\alpha 4\beta 7$ integrin-binding portion is a mature protein.

128. The fusion protein of Claim 126 wherein said $\alpha 4\beta 7$ integrin-binding portion is the complete extracellular domain of the polypeptide shown in Figure 1 (SEQ ID NO:2).

129. The fusion protein of Claim 126 wherein said $\alpha 4\beta 7$ integrin-binding portion consists of the two amino-terminal immunoglobulin domains of the polypeptide shown in Figure 1 (SEQ ID NO:2).

130. The fusion protein of Claim 126 further comprising a second moiety, wherein said second moiety is at least a portion of an immunoglobulin chain.

131. A fusion protein comprising a primate MAdCAM moiety, wherein said primate MAdCAM moiety has binding affinity for $\alpha 4\beta 7$ integrin and comprises an amino acid sequence selected from the group consisting of SEQ ID NO:4 and the amino acid sequence of an $\alpha 4\beta 7$ integrin-binding portion of the polypeptide shown in Figure 2 (SEQ

ID NO:4), wherein said $\alpha 4\beta 7$ integrin-binding portion comprises the N-terminal immunoglobulin-like domain.

132. The fusion protein of Claim 131 wherein said $\alpha 4\beta 7$ integrin-binding portion is a mature protein.

133. The fusion protein of Claim 131 wherein said $\alpha 4\beta 7$ integrin-binding portion consists of the complete extracellular domain of the polypeptide shown in Figure 2 (SEQ ID NO:4).

134. The fusion protein of Claim 131 wherein said $\alpha 4\beta 7$ integrin-binding portion is the two amino-terminal immunoglobulin domains of the polypeptide shown in Figure 2 (SEQ ID NO:4).

135. The fusion protein of Claim 131 further comprising a second moiety, wherein said second moiety is at least a portion of an immunoglobulin chain.

136. A fusion protein comprising a naturally occurring primate MAdCAM, wherein said naturally occurring primate MAdCAM binds $\alpha 4\beta 7$ integrin and has at least about 90% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.

137. The fusion protein of Claim 136, comprising a first moiety and a second moiety, wherein said first moiety is the naturally occurring primate MAdCAM and said second moiety is at least a portion of an immunoglobulin chain.

138. The fusion protein of Claim 137, wherein said first moiety is joined at its C-terminal end to the N-terminal end of the second moiety.

139. The fusion protein of Claim 137, wherein the second moiety is at least a portion of an immunoglobulin heavy chain constant region.

140. The fusion protein of Claim 139, wherein the immunoglobulin heavy chain is of the IgG class.

141. The fusion protein of Claim 139, wherein the second moiety comprises hinge, CH2 and CH3 domains of an immunoglobulin heavy chain.

142. A hybrid immunoglobulin comprising a fusion protein of Claim 137.

143. The hybrid immunoglobulin of Claim 142, wherein said hybrid immunoglobulin is a homodimer.

144. The fusion protein of Claim 136 wherein said primate MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 or a nucleic acid that shares at least about 90% nucleotide sequence similarity with SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

145. A fusion protein comprising an α4β7 integrin-binding fragment of a naturally occurring primate MAdCAM, wherein said naturally occurring primate MAdCAM has at least about 90% amino acid sequence similarity to SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6, and said α4β7 integrin-binding fragment comprises at least one immunoglobulin-like domain of said primate MAdCAM.

146. The fusion protein of Claim 145, wherein said α4β7 integrin-binding fragment is selected from the group consisting of a fragment comprising the extracellular domain of said naturally occurring primate MAdCAM and a fragment comprising the two N-terminal immunoglobulin domains of said naturally occurring primate MAdCAM.

147. A hybrid immunoglobulin comprising a fusion protein of Claim 145.

148. The hybrid immunoglobulin of Claim 147, wherein said hybrid immunoglobulin is a homodimer.

149. A fusion protein comprising a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4.

150. The fusion protein of Claim 149 wherein said human MAdCAM is encoded by SEQ ID NO:1, SEQ ID NO:3 or a nucleic acid that shares at least about 90% nucleotide sequence similarity with SEQ ID NO:1 or SEQ ID NO:3.

152. A hybrid immunoglobulin comprising a fusion protein of Claim 149.

153. The hybrid immunoglobulin of Claim 152, wherein said hybrid immunoglobulin is a homodimer.

154. A fusion protein comprising an $\alpha4\beta7$ integrin-binding fragment of a naturally occurring human MAdCAM, wherein said naturally occurring human MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to SEQ ID NO:2 or SEQ ID NO:4, and said $\alpha4\beta7$ integrin-binding fragment comprises the two N-terminal immunoglobulin-like domains of said human MAdCAM.

155. A hybrid immunoglobulin comprising a fusion protein of Claim 154.

156. The hybrid immunoglobulin of Claim 155, wherein said hybrid immunoglobulin is a homodimer.

157. A fusion protein comprising a primate MAdCAM or $\alpha4\beta7$ integrin-binding fragment thereof, wherein said primate MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and said $\alpha4\beta7$ integrin-binding fragment comprises at lease one immunoglobulin-like domain of said primate MAdCAM.

158. The fusion protein of Claim 157, wherein said primate MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to SEQ ID NO:2.

159. The fusion protein of Claim 157, wherein said primate MAdCAM binds $\alpha4\beta7$ integrin and has at least about 90% amino acid sequence similarity to SEQ ID NO:4.

160. The fusion protein of Claim 157, wherein said $\alpha4\beta7$ integrin-binding fragment is selected from the group consisting of a fragment comprising the extracellular domain of said primate MAdCAM and a fragment comprising the two N-terminal immunoglobulin domains of said primate MAdCAM.

## EVIDENCE APPENDIX

1. Declaration Under 37 CFR § 1.132

A copy of a Declaration of Michael J. Briskin, Ph.D. that was originally filed in U.S. Patent Application No. 08/523,004 was entered into the record on November 19, 2001.

An unexecuted copy of the Declaration was originally filed in U.S. Patent Application No. 08/523,004 on October 5, 1998, and the executed Declaration was filed in U.S. Patent Application No. 08/523,004 on October 26, 1998. Appellant's copy of the executed Declaration and transmittal paper that were filed in U.S. Patent Application No. 08/523,004 does not include page 1 of the Declaration.

Copies of both the unexecuted Declaration and the executed Declaration and transmittal paper that were filed in the subject application on November 19, 2001 are provided herewith.

2. Shyjan *et al.*, "Human Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) Demonstrates Structural and Functional Similarities to the $\alpha 4\beta 7$-Integrin Binding Domains of Murine MAdCAM-1, but Extreme Divergence of Mucin-Like Sequences," *J. Immunol., 156*:2851-2857 (1996).

Shyjan *et al.* was provided as Reference AX4 with the Third Supplemental Information Disclosure Statement entered into the record on June 4, 1999.

RELATED PROCEEDINGS APPENDIX

- NONE -

K:\ACOLLINS\LKS9404A\TRANS\ ´001
DEF:HEW:AJC:kfd
October 22, 1998

OIPE
AUG 16 2006
PATENT & TRADEMARK OFFICE

P TENT APPLICATION
Attorney' ocket No.: LKS94-04A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Michael J. Briskin, Douglas J. Ringler, Dominic Picarella and Walter Newman

Application No.: 08/523,004 Group: 1644

Filed: September 1, 1995 Examiner: R. Schwadron

For: NOVEL MUCOSAL VASCULAR ADDRESSINS

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231

on 10-22-98 Karen DiRocco
Date Signature

Karen DiRocco
Typed or printed name of person signing certificate

**COPY**

TRANSMITTAL OF EXECUTED DECLARATION UNDER 37 C.F.R §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an executed Declaration under 37 C.F.R. §1.132. An unexecuted Declaration under 37 C.F.R. §1.132 and an Amendment A were mailed to the U.S. Patent and Trademark Office on September 30, 1998 for filing in the referenced application. As indicated in Amendment A, the executed Declaration would be filed in the referenced application upon receipt.

The executed Declaration is identical to the unexecuted Declaration, except that the term "loopes" on page 4, line 22 of the unexecuted Declaration has been corrected to recite "loops" in the executed Declaration. Applicants respectfully request that the executed Declaration be entered in the referenced application.

Respectfully submitted,
HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Anne J. Collins

Anne J. Collins
Registration No. 40,564
Telephone (781) 861-6240
Facsimile (781) 861-9540

Lexington, Massachusetts 02421-4799
Dated: October 22, 1998

OIPE IAP17 AUG 16 2006 PATENT & TRADEMARK OFFICE

2. In 1993, upon completion of my postdoctoral research at Amgen, Inc. and Stanford University School of Medicine, Department of Pathology, I accepted a position at LeukoSite, Inc. I am presently employed as a Senior Research Scientist by LeukoSite, Inc., Assignee of the subject application.

3. I am familiar with the subject application and the invention claimed therein.

4. Since the filing of the subject application, additional studies of human MAdCAM-1 have been completed at LeukoSite, which support the subject application. In particular, a series of point mutations in the human MAdCAM-1 amino acid sequence were prepared. The additional work described herein was performed by Nancy Green and Josh Rosebrook, employees of LeukoSite, Inc., working under my direction and supervision.

5. As described herein, structure/function studies of human MAdCAM-1 have been carried out. Variants of human MAdCAM-1 were generated in order to define sites required for binding to the $\alpha4\beta7$ integrin. 31 different point mutants of human MAdCAM-1 were produced. The function of the resulting variants was assessed using an adhesion assay which monitors binding to the $\alpha4\beta7$ integrin. 14 of the 31 point mutants assessed displayed mean binding to $\alpha4\beta7$ at levels between 80% - 100% of the control in the presence of $Mn^{2+}$ (The Table).

Mutagenesis

Human MAdCAM-1 mutants were prepared as soluble immunoglobulin fusion proteins (also referred to as "HuMAdIg receptor" proteins) consisting of the entire extracellular domain of human MAdCAM-1 fused to the entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al.*, *J. Immunol.*, *159*:1497-1505 (1997)). A series of point mutants of a construct encoding a soluble human MAdCAM-1-Ig fusion protein (a wild type HuMAdIg receptor) were made using the QuikChange Mutagenesis Kit and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Alanine was substituted in all cases except for additional CD Loop mutations, L45R and D46E. Mutagenic primers contained 10-16 bp of template sequence adjacent to the changed bases

(e.g., the sense primer for D46A reads 5'-CGG GGC CTG GcC ACC AGC CTG G-3'). All mutated regions were sequenced using the ThermoSequenase Kit (Amersham Life Science, Inc., Cleveland, OH).

Preparation of Fusion Proteins

Soluble chimeric proteins were produced by transient transfection of CHO/P cells. CHO/P cells were transfected with constructs encoding mutant MAdCAM-1-Ig fusions proteins. CHO/P cells were plated at 1 x$10^6$ cells/10 cm plate (Falcon), and incubated overnight at 37°C. Media was removed and cells were rinsed with 2 ml Opti-MEM-1 (GIBCO, Life Technologies, Gaithersburg, MD). LIPOFECTAMINE Reagent (GIBCO) (60 $\mu$l/plate) was mixed with 10 $\mu$g plasmid DNA in 1.6 mls Opti-MEM-1, incubated 25 minutes at ambient temperature and subsequently added to cells with 6.4 ml additional Opti-MEM-1. After 2.25 hours incubation at 37°C, an equal volume (8 mls) of alpha-MEM/20% fetal bovine serum (GIBCO) was added to cells for overnight incubation. Approximately 18 hours after transfection, transfection media was replaced with 10 mls of MEM-$\alpha$ media and then incubated 72-96 hours before collection for purification. Chromatography columns (BioRad) were packed with 3 mls of Protein A Sepharose Fast Flow resin (Pharmacia Biotech) and pre-equilibrated with phosphate buffered saline (PBS). Mutant Ig chimera supernatanants were diluted 1:1 in PBS, filtered and applied to columns by gravity flow. Columns were washed with 60 mls PBS, then material eluted by addition of 4 ml of 0.1 M Citrate pH 3.5, neutralized and dialyzed against PBS.

Soluble proteins were quantitated by ELISA. 96-well plates (NUNC Maxisorb) were pre-coated with anti-human IgG (Jackson Immuno-Research, West Grove, PA), 10 µg/ml, 50 µl/well in carbonate buffer pH 9.5 and incubated overnight at 4°C, then blocked with PBS/5% gelatin for 2 hours at 37°C. Samples and standard (human IgG1) were diluted in 1X THST (1mM glycine, 0.5 M NaCl, 50 mM TRIZMA base pH 8.0, 0.05% TWEEN 20), applied to plates, and incubated for 2 hours at room temperature. Plates were rinsed with 1X THST. Detection was via peroxidase-conjugated Goat-anti mouse IgG (Jackson), 1:4000 in 1X THST, 50 µl/well. Assays were visualized with OPD (Sigma) and absorbance was read at 490 nm.

Adhesion Assays

Cell lines used for functional adhesion assays were CHO/P (Heffernan, M. and Dennis, J.D., *Nucl. Acids Res., 19*:85 (1991)), RPMI 8866 which only express α4β7 (Erle, D.J., *et al., J. Immunol., 153*:517-528 (1994)), and Ramos which express only α4β1 (ATCC). For adhesion assays with soluble huMAdIg receptors, proteins were plated onto wells of a 96-well RIA plate (Costar, Cambridge, MA) at 0.2 μg/ml in 50 μl phosphate buffered saline. Plates were incubated overnight a 4°C, and blocked for 2 hours at 37°C (PBS, 10% calf serum, Gibco). Labeled RPMI 8866 cells were added to each well at 1.25 x $10^6$/well, then plates were incubated for 30 minutes at room temperature. Plates were washed for 2 cycles on a Microplate Autowasher (Biotek Instruments), then fluorescence was quantitated. Each sample was assayed in triplicate and all assays were performed at least three times.

Results

Mutagenesis of Individual Human MAdCAM-1 Residues

In order to further define the α4β7 binding site in human MAdCAM-,1 a series of single amino acid substitutions in regions that were predicted to be important for IgCAM/integrin interactions based on other studies, were generated. The study focused primarily on selected residues in interstrand loops in both domains 1 and 2 of human MAdCAM. The approximate locations of these loops were based upon the crystal structure of human VCAM-1. The locations that were targeted aligned with interchain loops of other IgCAMs that have been shown to be important in integrin recognition, namely the CD and EF loops of domain 1 and the C'E and FG loops of domain 2 (Wang, J-H., *et al., Proc. Natl. Acad. Sci. USA, 92*:5714-5718 (1995); Jones, E.Y., *et al., Nature, 373*:539-544 (1995)). Additionally, some of these regions were shown to be important for ICAM-1, ICAM-3 and VCAM-1 mediated adhesion (Staunton, D.E., *et al., Cell, 61*:243 (1990); Osborn, L., *et al., J. Cell Biol., 124*:601 (1994); Holness, C.A., *et al., J. Biol. Chem., 270*:877 (1995)). In most instances, alanine substitution was used, and most amino acids in each region were targeted for mutation with the exception of small, hydrophobic resides. As noted above, human MAdCAM-1 mutants were prepared as soluble Ig-fusion proteins consisting of the entire extracellular domain of human MAdCAM-1 fused to the

entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al.*, *J. Immunol.*, *157*:1497-1505 (1997)). Recombinant soluble huMAdIg receptor proteins were transiently produced in CHO/P cells, purified by protein A chromatography, and quantitated in an IgG based ELISA to ensure that similar amounts of receptors were used in each experiment.

Adhesion was compared to binding to wild type MAdCAM-Ig fusion protein in an RPMI 8866 cell binding assay, initially in the absence of activating stimuli. Alanine substitution of R39, L41, D42, T43, R70, E148, E151, E152, E157 and H195 reduced adhesion to less than 25% adhesion as compared to adhesion of wild-type MAdCAM-1 (the Table). Additionally, a conservative change of D42E essentially abolished binding as adhesion was reduced to only 4.3% of normal. Moderate effects (25-50% adhesion) on binding were observed with S55 and E149, while mild effects (50-75% adhesion) were seen with L64, E145, Q147 and D156. All other mutations did not affect binding significantly (75-100% adhesion), with the exception of alanine substitution of R60 and E192 which resulted in significant increases in binding (the Table).

To further assess the severity of the mutations tested, the assays were repeated in the presence of 1 mM $Mn^{2+}$, which has been shown to fully activate integrins, including $\alpha 4\beta 7$ (Berlin, C., *et al.*, *Cell*, *74*:185-195 (1993); Briskin, M.J., *et al.*, *J. Immunol.*, *156*:719-726 (1996)). Under these conditions, mutation of only three residues (L41, D42 and E148) yielded mutants which display adhesion at levels less than 25% of the adhesion of wild type MAdCAM-1. Three other residues, R70, E152 and E157 were also moderately (25%-50% adhesion) affected in the presence of $Mn^{2+}$. Additionally, the two mutations (R60 and E192) that originally showed increased binding displayed binding at similar levels to native MAdCAM-1 under these conditions.

The results of performing the assays with activated integrin were similar to looking at adhesion of the mutant receptors at higher concentrations. The severe mutations such as L41A and D42A or E could not be rescued by using higher receptor densities, while the R70A mutation was only partially rescued at receptor concentrations 4-fold higher than in the standard assays. Conversely, many of the mutations in the DE loop of domain 2 were restored to levels closer to native MAdCAM at higher receptor avidity, indicating that these mutations might have different mechanistic effects.

Discussion

Site-directed mutagenesis was performed to define critical α4β7 binding residues in both Ig domains 1 and 2. The results are consistent with data obtained for other Ig-CAMS showing that an essential contact site resides in the CD loop in domain 1, the most significant residues being L41 and D42 (the Table). Alanine substitution in either of these sites abolishes integrin interactions, even when α4β7 is fully activated by $Mn^{2+}$. The absolute requirement for a defined structure of the CD loop is illustrated by the fact that a conservative substitution, D42E, also results in a complete abrogation of binding (without $Mn^{2+}$). Additionally, we also found that R39A resulted in significantly decreased adhesion, while the analogous residue in murine MAdCAM-1 R38 was unaffected by mutation to alanine (Viney, J.L., *et al., J. Immunol., 157*:2488-2497 (1997)). The discrepancy between this data for human R39A versus murine R38A MAdCAM-1 may reflect a technical difference in the assays performed or may reflect structural distinctions due to the weak sequence conservation (57% identity) between the murine and human homologs (Shyjan, A.M., *et al., J. Immunol., 156*:2851-2857 (1996)).

TABLE

| Mutant | without $Mn^{++}$ Mean | SDEV | with $Mn^{++}$ Mean | SDEV |
|---|---|---|---|---|
| R39A | 20.48 | 8.24 | 62.25 | 3.14 |
| L41A | 0.25 | 0.50 | 10.88 | 3.39 |
| D42A | 1.38 | 2.75 | 1.5 | 1.24 |
| D42E | 4.3 | 8.53 | 23.1 | 5.08 |
| T43A | 5.08 | 4.44 | 54.4 | 3.56 |
| S55A | 27.94 | 25.21 | 88.65 | 5.17 |
| R60A | 224.68 | 27.10 | 97.4 | 5.89 |
| N61A | 118.23 | 38.56 | 88.2 | 6.23 |
| L64A | 59.8 | 19.11 | 81.9 | 3.20 |
| S65A | 101.2 | 31.20 | 84.4 | 4.30 |
| T69A | 144.73 | 38.07 | 83.93 | 7.25 |
| R70A | 1.05 | 1.22 | 48.4 | 4.3 |
| P144A | 102.93 | 17.12 | 80.23 | 8.55 |
| E145A | 67.83 | 30.35 | 80.7 | 6.73 |
| Q147A | 73.8 | 14.00 | 71.5 | 6.8 |
| E148A | 1.43 | 1.67 | 22.6 | 5.05 |
| E149A | 37.9 | 23.80 | 62.9 | 9.20 |
| E150A | 116.85 | 23.05 | 82.58 | 2.22 |
| E151A | 6.18 | 7.15 | 67.3 | 0.42 |
| E152A | 0.8 | 0.98 | 29.2 | 6.08 |
| P153A | 88.38 | 38.41 | 86.73 | 8.92 |
| Q154A | 91.04 | 34.00 | 72.1 | 9.10 |
| D156A | 56.08 | 6.78 | 65.78 | ##### |
| E157A | 8.6 | 5.27 | 49.15 | ##### |
| D158A | 86.5 | 11.90 | 83.68 | 2.74 |
| R162A | 97.18 | 15.60 | 75.48 | 6.16 |
| R187 | 92.83 | 17.33 | 85.23 | 3.54 |
| E192A | 147.95 | 4.14 | 85.65 | 3.80 |
| S194A | 74.55 | 28.24 | 80.15 | 5.49 |
| H195A | 22.45 | 10.31 | 66.78 | 6.88 |
| H196A | 86.03 | 15.91 | 71.43 | 4.57 |

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Michael J. Briskin | 10-20-98
Michael J. Briskin | Date

LKS9404A.132
K:\ACOLLINS\AJC
DEB/HEW/AJC/lbj
September 30, 1998

OIPE
AUG 16 2006
PATENT & TRADEMARK OFFICE

P. ENT APPLICATION
DOCKET NO.: LKS94-04A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| Applicants: | Michael J. Briskin, Douglas J. Ringler, Dominic Picarella and Walter Newman | | |
| Application No.: | 08/523,004 | Group Art Unit: | 1644 |
| Filed: | September 1, 1995 | Examiner: | R. Schwadron |
| For: | NOVEL MUCOSAL VASCULAR ADDRESSINS | | |

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231

on 9-30-98 (Date) Karen DiRocco (Signature)

Karen DiRocco

Typed or printed name of person signing certificate

COPY

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents

Washington, DC 20231

Sir:

I, Michael J. Briskin, Ph.D., of 28 Harbell Street, Lexington, Massachusetts 02421, hereby declare and state that:

1. I received a Bachelor of Science in Biology in 1979 from The University of California, Los Angeles, and a doctorate in Molecular Biology in 1988 from the University of California, Los Angeles.

2. In 1993, upon completion of my postdoctoral research at Amgen, Inc. and Stanford University School of Medicine, Department of Pathology, I accepted a position at LeukoSite, Inc. I am presently employed as a Senior Research Scientist by LeukoSite, Inc., Assignee of the subject application.

3. I am familiar with the subject application and the invention claimed therein.

4. Since the filing of the subject application, additional studies of human MAdCAM-1 have been completed at LeukoSite, which support the subject application. In particular, a series of point mutations in the human MAdCAM-1 amino acid sequence were prepared. The additional work described herein was performed by Nancy Green and Josh Rosebrook, employees of LeukoSite, Inc., working under my direction and supervision.

5. As described herein, structure/function studies of human MAdCAM-1 have been carried out. Variants of human MAdCAM-1 were generated in order to define sites required for binding to the $\alpha4\beta7$ integrin. 31 different point mutants of human MAdCAM-1 were produced. The function of the resulting variants was assessed using an adhesion assay which monitors binding to the $\alpha4\beta7$ integrin. 14 of the 31 point mutants assessed displayed mean binding to $\alpha4\beta7$ at levels between 80% - 100% of the control in the presence of $Mn^{2+}$ (The Table).

<u>Mutagenesis</u>

Human MAdCAM-1 mutants were prepared as soluble immunoglobulin fusion proteins (also referred to as "HuMAdIg receptor" proteins) consisting of the entire extracellular domain of human MAdCAM-1 fused to the entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al., J. Immunol., 159*:1497-1505 (1997)). A series of point mutants of a construct encoding a soluble human MAdCAM-1-Ig fusion protein (a wild type HuMAdIg receptor) were made using the QuikChange Mutagenesis Kit and *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Alanine was substituted in all cases except for additional CD Loop mutations, L45R and D46E.

Mutagenic primers contained 10-16 bp of template sequence adjacent to the changed bases (e.g., the sense primer for D46A reads 5'-CGG GGC CTG GcC ACC AGC CTG G-3'). All mutated regions were sequenced using the ThermoSequenase Kit (Amersham Life Science, Inc., Cleveland, OH).

Preparation of Fusion Proteins

Soluble chimeric proteins were produced by transient transfection of CHO/P cells. CHO/P cells were transfected with constructs encoding mutant MAdCAM-1-Ig fusions proteins. CHO/P cells were plated at 1 $\times 10^6$ cells/10 cm plate (Falcon), and incubated overnight at 37°C. Media was removed and cells were rinsed with 2 ml Opti-MEM-1 (GIBCO, Life Technologies, Gaithersburg, MD). LIPOFECTAMINE Reagent (GIBCO) (60 $\mu$l/plate) was mixed with 10 $\mu$g plasmid DNA in 1.6 mls Opti-MEM-1, incubated 25 minutes at ambient temperature and subsequently added to cells with 6.4 ml additional Opti-MEM-1. After 2.25 hours incubation at 37°C, an equal volume (8 mls) of alpha-MEM/20% fetal bovine serum (GIBCO) was added to cells for overnight incubation. Approximately 18 hours after transfection, transfection media was replaced with 10 mls of MEM-$\alpha$ media and then incubated 72-96 hours before collection for purification. Chromatography columns (BioRad) were packed with 3 mls of Protein A Sepharose Fast Flow resin (Pharmacia Biotech) and pre-equilibrated with phosphate buffered saline (PBS). Mutant Ig chimera supernatanants were diluted 1:1 in PBS, filtered and applied to columns by gravity flow. Columns were washed with 60 mls PBS, then material eluted by addition of 4 ml of 0.1 M Citrate pH 3.5, neutralized and dialyzed against PBS.

Soluble proteins were quantitated by ELISA. 96-well plates (NUNC Maxisorb) were pre-coated with anti-human IgG (Jackson Immuno-Research, West Grove, PA), 10 $\mu$g/ml, 50 $\mu$l/well in carbonate buffer pH 9.5 and incubated overnight at 4°C, then blocked with PBS/5% gelatin for 2 hours at 37°C. Samples and standard (human IgG1) were diluted in 1X THST (1mM glycine, 0.5 M NaCl, 50 mM TRIZMA base pH 8.0, 0.05% TWEEN 20), applied to plates, and incubated for 2 hours at room temperature. Plates were rinsed with 1X THST. Detection was via peroxidase-conjugated Goat-anti mouse IgG

(Jackson), 1:4000 in 1X THST, 50 µl/well. Assays were visualized with OPD (Sigma) and absorbance was read at 490 nm.

Adhesion Assays

Cell lines used for functional adhesion assays were CHO/P (Heffernan, M. and Dennis, J.D., *Nucl. Acids Res., 19*:85 (1991)), RPMI 8866 which only express α4β7 (Erle, D.J., *et al.*, *J. Immunol., 153*:517-528 (1994)), and Ramos which express only α4β1 (ATCC). For adhesion assays with soluble huMAdIg receptors, proteins were plated onto wells of a 96-well RIA plate (Costar, Cambridge, MA) at 0.2 µg/ml in 50 µl phosphate buffered saline. Plates were incubated overnight a 4°C, and blocked for 2 hours at 37°C (PBS, 10% calf serum, Gibco). Labeled RPMI 8866 cells were added to each well at 1.25 x $10^6$/well, then plates were incubated for 30 minutes at room temperature. Plates were washed for 2 cycles on a Microplate Autowasher (Biotek Instruments), then fluorescence was quantitated. Each sample was assayed in triplicate and all assays were performed at least three times.

Results

Mutagenesis of Individual Human MAdCAM-1 Residues

In order to further define the α4β7 binding site in human MAdCAM-,1 a series of single amino acid substitutions in regions that were predicted to be important for IgCAM/integrin interactions based on other studies, were generated. The study focused primarily on selected residues in interstrand loops in both domains 1 and 2 of human MAdCAM. The approximate locations of these loopes were based upon the crystal structure of human VCAM-1. The locations that were targeted aligned with interchain loops of other IgCAMs that have been shown to be important in integrin recognition, namely the CD and EF loops of domain 1 and the C'E and FG loops of domain 2 (Wang, J-H., *et al.*, *Proc. Natl. Acad. Sci. USA, 92*:5714-5718 (1995); Jones, E.Y., *et al.*, *Nature, 373*:539-544 (1995)). Additionally, some of these regions were shown to be important for ICAM-1, ICAM-3 and VCAM-1 mediated adhesion (Staunton, D.E., *et al.*, *Cell, 61*:243 (1990); Osborn, L., *et al.*, *J. Cell Biol., 124*:601 (1994); Holness, C.A., *et al.*, *J. Biol.*

*Chem.*, *270*:877 (1995)). In most instances, alanine substitution was used, and most amino acids in each region were targeted for mutation with the exception of small, hydrophobic resides. As noted above, human MAdCAM-1 mutants were prepared as soluble Ig-fusion proteins consisting of the entire extracellular domain of human MAdCAM-1 fused to the entire constant region of an Fc mutated human IgG1 heavy chain (Tidswell, M., *et al.*, *J. Immunol.*, *157*:1497-1505 (1997)). Recombinant soluble huMAdIg receptor proteins were transiently produced in CHO/P cells, purified by protein A chromatography, and quantitated in an IgG based ELISA to ensure that similar amounts of receptors were used in each experiment.

Adhesion was compared to binding to wild type MAdCAM-Ig fusion protein in an RPMI 8866 cell binding assay, initially in the absence of activating stimuli. Alanine substitution of R39, L41, D42, T43, R70, E148, E151, E152, E157 and H195 reduced adhesion to less than 25% adhesion as compared to adhesion of wild-type MAdCAM-1 (the Table). Additionally, a conservative change of D42E essentially abolished binding as adhesion was reduced to only 4.3% of normal. Moderate effects (25-50% adhesion) on binding were observed with S55 and E149, while mild effects (50-75% adhesion) were seen with L64, E145, Q147 and D156. All other mutations did not affect binding significantly (75-100% adhesion), with the exception of alanine substitution of R60 and E192 which resulted in significant increases in binding (the Table).

To further assess the severity of the mutations tested, the assays were repeated in the presence of 1 mM $Mn^{2+}$, which has been shown to fully activate integrins, including $\alpha 4\beta 7$ (Berlin, C., *et al.*, *Cell*, *74*:185-195 (1993); Briskin, M.J., *et al.*, *J. Immunol.*, *156*:719-726 (1996)). Under these conditions, mutation of only three residues (L41, D42 and E148) yielded mutants which display adhesion at levels less than 25% of the adhesion of wild type MAdCAM-1. Three other residues, R70, E152 and E157 were also moderately (25%-50% adhesion) affected in the presence of $Mn^{2+}$. Additionally, the two mutations (R60 and E192) that originally showed increased binding displayed binding at similar levels to native MAdCAM-1 under these conditions.

The results of performing the assays with activated integrin were similar to looking at adhesion of the mutant receptors at higher concentrations. The severe mutations such as

L41A and D42A or E could not be rescued by using higher receptor densities, while the R70A mutation was only partially rescued at receptor concentrations 4-fold higher than in the standard assays. Conversely, many of the mutations in the DE loop of domain 2 were restored to levels closer to native MAdCAM at higher receptor avidity, indicating that these mutations might have different mechanistic effects.

Discussion

Site-directed mutagenesis was performed to define critical $\alpha4\beta7$ binding residues in both Ig domains 1 and 2. The results are consistent with data obtained for other Ig-CAMS showing that an essential contact site resides in the CD loop in domain 1, the most significant residues being L41 and D42 (the Table). Alanine substitution in either of these sites abolishes integrin interactions, even when $\alpha4\beta7$ is fully activated by $Mn^{2+}$. The absolute requirement for a defined structure of the CD loop is illustrated by the fact that a conservative substitution, D42E, also results in a complete abrogation of binding (without $Mn^{2+}$). Additionally, we also found that R39A resulted in significantly decreased adhesion, while the analogous residue in murine MAdCAM-1 R38 was unaffected by mutation to alanine (Viney, J.L., *et al.*, *J. Immunol.*, *157*:2488-2497 (1997)). The discrepancy between this data for human R39A versus murine R38A MAdCAM-1 may reflect a technical difference in the assays performed or may reflect structural distinctions due to the weak sequence conservation (57% identity) between the murine and human homologs (Shyjan, A.M., *et al.*, *J. Immunol.*, *156*:2851-2857 (1996)).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Michael J. Briskin

Date

TABLE

| Mutant | without Mn++ Mean | SDEV | with Mn++ Mean | SDEV |
|---|---|---|---|---|
| R39A | 20.48 | 8.24 | 62.25 | 3.14 |
| L41A | 0.25 | 0.50 | 10.88 | 3.39 |
| D42A | 1.38 | 2.75 | 1.5 | 1.24 |
| D42E | 4.3 | 8.53 | 23.1 | 5.08 |
| T43A | 5.08 | 4.44 | 54.4 | 3.56 |
| S55A | 27.94 | 25.21 | 88.65 | 5.17 |
| R60A | 224.68 | 27.10 | 97.4 | 5.89 |
| N61A | 118.23 | 38.56 | 88.2 | 6.23 |
| L64A | 59.8 | 19.11 | 81.9 | 3.20 |
| S65A | 101.2 | 31.20 | 84.4 | 4.30 |
| T69A | 144.73 | 38.07 | 83.93 | 7.25 |
| R70A | 1.05 | 1.22 | 48.4 | 4.3 |
| P144A | 102.93 | 17.12 | 80.23 | 8.55 |
| E145A | 67.83 | 30.35 | 80.7 | 6.73 |
| Q147A | 73.8 | 14.00 | 71.5 | 6.8 |
| E148A | 1.43 | 1.67 | 22.6 | 5.05 |
| E149A | 37.9 | 23.80 | 62.9 | 9.20 |
| E150A | 116.85 | 23.05 | 82.58 | 2.22 |
| E151A | 6.18 | 7.15 | 67.3 | 0.42 |
| E152A | 0.8 | 0.98 | 29.2 | 6.08 |
| P153A | 88.38 | 38.41 | 86.73 | 8.92 |
| Q154A | 91.04 | 34.00 | 72.1 | 9.10 |
| D156A | 56.08 | 6.78 | 65.78 | #### |
| E157A | 8.6 | 5.27 | 49.15 | #### |
| D158A | 86.5 | 11.90 | 83.68 | 2.74 |
| R162A | 97.18 | 15.60 | 75.48 | 6.16 |
| R187 | 92.83 | 17.33 | 85.23 | 3.54 |
| E192A | 147.95 | 4.14 | 85.65 | 3.80 |
| S194A | 74.55 | 28.24 | 80.15 | 5.49 |
| H195A | 22.45 | 10.31 | 66.78 | 6.88 |
| H196A | 86.03 | 15.91 | 71.43 | 4.57 |

# Human Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) Demonstrates Structural and Functional Similarities to the $\alpha_4\beta_7$-Integrin Binding Domains of Murine MAdCAM-1, but Extreme Divergence of Mucin-Like Sequences[1]

Anne M. Shyjan,* Monica Bertagnolli,† Christopher J. Kenney,* and Michael J. Briskin[2]*

The mucosal vascular addressin, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), is an Ig family adhesion receptor preferentially expressed by venular endothelial cells at sites of lymphocyte extravasation in murine mucosal lymphoid tissues and lamina propria. MAdCAM-1 specifically binds both human and mouse lymphocytes that express the homing receptor for Peyer's patches, the integrin $\alpha_4\beta_7$. Functional expression cloning was used to isolate a cDNA from a macaque mesenteric lymph node library that encodes the homologue to murine MAdCAM-1. The macaque cDNA was subsequently used to clone the human homologue as well. Expression of human MAdCAM-1 RNA is restricted to mucosal tissues, gut-associated lymphoid tissues and spleen. Human MAdCAM-1 selectively binds both murine and human lymphocyte cell lines expressing $\alpha_4\beta_7$. Human and macaque MAdCAM-1 have two Ig-like domains that are similar to the two amino-terminal integrin binding domains of murine MAdCAM-1. The conservation of sequences within the region homologous to the mucin/IgA domain of murine MAdCAM-1 is, however, much less apparent. These receptors exhibit considerable variation from murine MAdCAM-1 with respect to the length of the mucin-like sequence and the lack of a membrane proximal Ig (IgA-like) domain. The isolation of these different species of MAdCAM-1 demonstrates greater selective pressure for maintenance of amino acids involved in $\alpha_4\beta_7$ binding than those sequences presumably involved in the presentation of carbohydrates for selectin binding. *The Journal of Immunology,* 1996, 156: 2851–2857.

The specificity of lymphocyte homing to both normal tissues and sites of inflammation is the result of several adhesive and activating events that involve multiple receptors (1–5). Within lymph nodes and Peyer's patches (PPs), this process is exquisitely regulated by interactions of homing receptors on the surface of lymphocytes with endothelial cells lining the post capillary venules, most notably high endothelial venules (HEV) (4, 5). In the mouse, lymphocyte binding to PPs involves a single chain 60-kDa glycoprotein, the mucosal vascular addressin, MAdCAM-1 (6, 7). Murine MAdCAM-1 is expressed in mesenteric lymph nodes (MLN), lamina propria of the small and large intestine, and the lactating mammary gland (4, 5). MAdCAM-1 expression can be induced in murine endothelial cells in vitro by treatment with pro-inflammatory cytokines (8). An in vivo role in inflammation has also been suggested by increased expression of MAdCAM-1 on HEV-like vessels in the chronically inflamed pancreas of the nonobese diabetic mouse (9, 10), in the central nervous system in chronic relapsing experimental allergic encephalomyelitis (11), and in lamina propria venules in murine models of inflammatory bowel disease (D. Ringler and D. Picarella, manuscript in preparation).

The lymphocyte integrin $\alpha_4\beta_7$ is the exclusive integrin receptor for MAdCAM-1 (12, 13). Abs to MAdCAM-1 or $\alpha_4\beta_7$ block lymphocyte binding to affinity-purified MAdCAM-1 or transfectants expressing MAdCAM-1 cDNA in vitro (6, 12, 13). These same Abs also inhibit localization of lymphocytes to PPs in short term in vivo homing experiments (14). Murine MAdCAM-1 binds only $\alpha_4\beta_7$-positive human lymphocyte cell lines and $\alpha_4\beta_7^{high}$ memory T cells (13). In vitro frozen section assays demonstrate that mucosal T cell lines and gut-derived immunoblasts bind to human appendix HEV and that adhesion is blocked only by Abs against the $\beta_7$-integrin (and not $\alpha_4\beta_1$) (13, 15) (see Footnote 4). As $\alpha_4\beta_7^{high}$ memory T cells appear to define a gut homing phenotype (13, 16) (see Footnote 4), these experiments indicate conservation of lymphocyte trafficking to mucosal tissues across species barriers.

Murine MAdCAM-1 is an Ig-like receptor (17). The two amino-terminal domains are homologous to its closest relatives among Ig-like adhesion receptors, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). The third (membrane proximal) domain,

*LeukoSite, Inc., Cambridge, MA 02142; and †Department of Surgery, New York Hospital/Cornell Medical Center, New York, NY 10021

Received for publication October 16, 1995. Accepted for publication February 8, 1996.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by American Cancer Society Grant CDA-95010 (to M.B.). The cDNA sequence presented is GenBank U43628.

[2] Address correspondence and reprint requests to Dr. Michael J. Briskin, LeukoSite, Inc., 215 First St., Cambridge, MA 02142.

[3] Abbreviations used in this paper: PP, Peyer's patches; HEV, high endothelial venules; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MLN, mesenteric lymph node; ICAM, ; VCAM-1, vascular cell adhesion molecule-1; S/T/P, serine/threonine/proline.

[4] Rott, L. S., M. J. Briskin, D. P. Andrew, E. L. Berg, and E. C. ~~Butcher. Hetero~~ geneity in homing receptor expression and MAdCAM-1 vs among human peripheral blood lymphocyte binding subs publication.

Copyright © 1996 by The American Association of Immunologists

while unrelated to adhesion receptors of this class, is homologous to another mucosal-related Ig family member, IgA. In addition to the three Ig-like domains, MAdCAM-1 has a S/T/P-rich mucin-like domain between the second and third Ig loops. Recent domain-swapping experiments demonstrate that the two amino-terminal Ig domains of MAdCAM-1 are sufficient for activation-independent adhesion to $\alpha_4\beta_7$ (18). MAdCAM-1, when expressed in mesenteric lymph nodes, can also present L-selectin binding carbohydrates associated with the peripheral node addressin epitope, MECA-79 (19). This modified species of MAdCAM-1 is functional, as lymphocytes can roll on this substrate via L-selectin in laminar flow assays. Initial contact of lymphocytes (rolling and tethering) with MAdCAM-1 can also be mediated by $\alpha_4\beta_7$ in addition to the role of this integrin in activation-dependent adhesion and arrest (20). In situ studies show that homing and extravasation of naive lymphocytes in PP HEV is mediated by both L-selectin and $\alpha_4\beta_7$ interactions with MAdCAM-1 and involves LFA-1 as well. In lamina propria venules, however, $\alpha_4\beta_7$/MAdCAM-1 interactions alone can mediate the adhesion of both preactivated and $\alpha_4\beta_7^{high}$ lymphocytes (20, 21). These experiments demonstrate that the multidomain structure of murine MAdCAM-1 is reflected at the level of functional diversity as well and further emphasize the critical role of this molecule in lymphocyte homing to mucosal sites.

Previous studies in our laboratory by both low stringency cross-hybridization to zoo blots and degenerate PCR based on murine MAdCAM-1 sequences, indicated that a human MAdCAM-1 gene sequence was not well conserved. The conservation $\alpha_4\beta_7$-dependent interactions thus served as the basis of our design of a functional cloning approach similar to that used to clone VCAM-1 (22). We describe the molecular cloning and functional characterization of the primate (macaque) and human homologues of MAdCAM-1. We demonstrate that while human and macaque MAdCAM-1 have retained sequences that are probably important for binding $\alpha_4\beta_7$, these receptors have significantly diverged from their murine counterpart.

## Materials and Methods

### Plasmids, mAbs, and cell lines

The following plasmids were used as controls in expression cloning and for functional adhesion assays: pSV-SPORT-1 (Life Technologies, Gaithersburg, MD) or pcDNA3 (Invitrogen, San Diego, CA) controls, murine MAdCAM-1 in pCDM8 (pCDMAD-7) (17), seven-domain human VCAM-1 (23) in pcDNA3 (pCD3VCAM), and human MAdCAM-1 in pcDNA3 (pCDhuMAd4). The mAbs used were anti-murine MAdCAM-1 MECA-367 (6), anti-human VCAM-1 2G7 (24), anti-murine $\beta$7 FIB 504 (25), anti-human $\alpha_4\beta_7$ ACT-1 (26), anti-human integrin $\beta_1$ (CD29, Becton Dickinson, San Jose, CA), and murine IgG1 and rat IgG2A as irrelevant controls. The cell lines used for expression cloning and functional adhesion assays were CHO/P (27), the murine T cell lymphoma TK1 (28), RPMI 8866 (13), and Jurkat (American Type Tissue Culture Collection, Rockville, MD).

### cDNA synthesis and library construction

mRNA was isolated from human and macaque mesenteric lymph nodes using standard procedures previously described (17). cDNA was synthesized using the Superscript lambda system in conjunction with either the λZiplox vector or the pSV-SPORT-1 vector (Life Technologies) essentially using their standard protocol. For the macaque expression library, only the largest (>1.5 kb) of cDNA were ligated into the pSV-SPORT-1 vector and plated in pools at a density of 1500 clones/plate on 100 LB agar plates with ampicillin (50 μg/ml). After incubation overnight, plasmid DNAs were purified from each plate individually by use of QIAprep spin columns (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

### Expression cloning

CHO/P cells were seeded into 24-well plates approximately 24 h before transfection at a density of 40,000 cells/well. DNAs are transiently transfected using LipofectAMINE reagent (Life Technologies) as follows. DNA, 200 ng (representing either a plasmid pool or purified control DNAs), and 2 μl of lipofectamine were used in a total volume of 40 μl in Opti-MEM 1; 200 μl of Opti-MEM 1 were then added after liposome formation, and the entire mixture was overlaid onto a well of CHO/P cells and returned to the incubator. After a 2.5-h incubation, 240 μl of MEM-$\alpha$ (Life Technologies) with 20% FCS were added to each well, and the cells were incubated for an additional 18 to 24 h. The medium was then changed to standard MEM-$\alpha$ with 10% FCS, and the adhesion assays were performed approximately 20 to 24 h later.

For the adhesion assays in the expression cloning screen, TK1 cells were resuspended at a density of $2 \times 10^6$/ml in an assay buffer consisting of HBSS (without $Ca^{2+}$ and $Mg^{2+}$) supplemented with 2% bovine calf serum, 20 mM HEPES (pH 7.3), 2 mM $Mg^{2+}$, and 2 mM $Ca^{2+}$. Each well transfected with a DNA pool was preincubated with 0.25 ml of a combined supernatant containing anti-human VCAM-1 mAb 2G7 (23) and anti-murine MAdCAM-1 MECA-367 (7). After incubation at 4°C for 15 min, 0.25 ml of the TK1 cell suspension ($5 \times 10^5$ TK1 cells) was added to each well, and incubation on a rocking platform was continued for an additional 30 min at 4°C. Plates were washed by gently inverting in a large beaker of PBS followed by inversion in a beaker of PBS with 1.5% glutaraldehyde for fixation for a minimum of 1 h. Wells were then examined microscopically (×10 objective) for rosetting of TK1 cells mediated by the pools of cDNA clones.

Pools yielding one or more TK1 rosettes were further subfractionated three times until individual colonies could be assayed, and the clones conferring adhesion of the TK1 cells were identified.

Functional adhesion assays with purified clones were similar to those performed in expression cloning with the following exception; as several wells were to be transfected for Ab inhibition studies, a master liposome mix with multiples of the wells to be transfected was first made for each plasmid. On the day of the assay, mAbs were incubated with cells at 20 μg/ml or supernatants (undiluted) at 4°C for 15 min before the start of the assay. Assays were fixed as described above and quantitated by counting four fields of both lymphocytes and CHO cells at ×10 magnification.

### Screening of human phage library and purification of human clones

Human phage cDNA libraries from MLN were made in the in λZiplox vector from Life Technologies. cDNA was synthesized as described above, ligated into the phage vector, and titrated on bacterial strain Y1090 (ZL). Duplicate filters from approximately 500,000 independent clones (50,000 clones/filter) were denatured using standard conditions, prehybridized, and subsequently hybridized with $^{32}$P-labeled full-length macaque MAdCAM-1 cDNA overnight at 55°C in a buffer previously described (30). Filters were subsequently washed at high stringency (65°C, 0.1× SSC and 0.1% SDS). Positive clones were plaque purified, and plasmid PZL1 containing the cDNA inserts was rescued using the CRE LOX recombination system described by Life Technologies. Rescued plasmids were then purified using Qiagen plasmid purification reagents.

### DNA sequencing

Plasmids were sequenced on both strands using oligonucleotide primers and the Sequenase 7-deaza-dGTP DNA sequencing kit with Sequenase version 2.0 T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH) and [$^{35}$S]dCTP (Amersham Life Science (Arlington Heights, IL) and New England Nuclear (Boston, MA)) while sequencing of GC-rich regions was facilitated by use of the $\delta$ TAQ sequencing kit (U.S. Biochemical Corp.) and [$\gamma$-$^{33}$P]ATP. Sequences were entered and analyzed using the Lasergene system (DNASTAR, Inc., Madison, WI).

### Northern blot analysis

The Northern blots used were human multiple tissue Northern blots I and II (Clontech, Palo Alto, CA). Hybridization was performed with ExpressHyb (Clontech) solution, using the manufacturer's instructions, except that a final wash at high stringency (0.1× SSC and 0.1% SDS, 65°C) for 30 min was added. cDNAs were labeled with [$\alpha$-$^{32}$P]dCTP by priming with random hexamers. After washing, filters were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) with an intensifying screen.

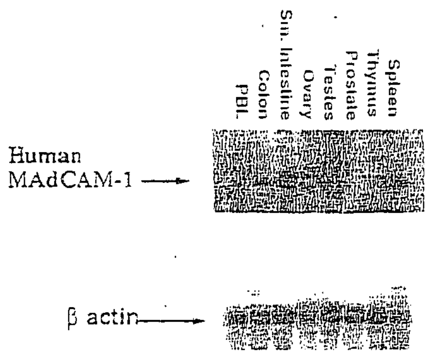


FIGURE 1. Human MAdCAM-1 RNA is only expressed in mucosal tissues. A Northern blot, consisting of 2 $\mu$g of the indicated mRNAs, was initially hybridized with the cDNA encoding human MAdCAM-1, washed at high stringency, and exposed for 48 h with an intensifying screen. After this exposure, the blot was stripped and rehybridized with a $\beta$-actin cDNA and exposed for 2 h.

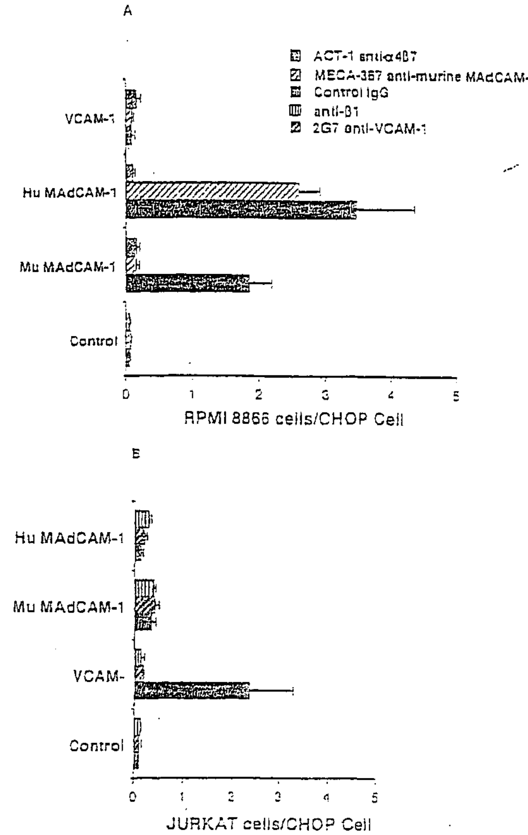


FIGURE 2. Human MAdCAM-1 only binds lymphocytes expressing $\alpha_4\beta_7$. *A*, RPMI 8866 cells bind CHO/P cells expressing murine and human MAdCAM-1, but fail to bind VCAM-1 or control transfectants. Abs and procedures are described in *Materials and Methods*. *B*, Jurkat cells bind VCAM-1 transfectants, but do not bind control, murine, or human MAdCAM-1 transfectants. For each assay, the number of lymphocytes bound per CHO/P cell was averaged as a minimum of four fields (×10 objective) with the SE. Results in each case are from one of three experiments performed with similar results.

## Results

### *Cloning of macaque and human MAdCAM-1*

Initial attempts to clone the human homologue to murine MAdCAM-1 by low stringency cross-hybridization suggested that nucleotide conservation between murine MAdCAM-1 and higher species was poor (not shown). As MAdCAM-1/$\alpha_4\beta_7$ interactions appear to be evolutionarily conserved (13, 15, 29) (see Footnote 4) (E. C. Butcher, unpublished observations), we set up a functional approach, whereby we could identify cDNAs that would confer adhesion of a target lymphocyte cell line that expresses high levels of $\alpha_4\beta_7$. As human mesenteric lymph nodes were scarce, we constructed an expression library from macaque MLNs. This library was transiently transfected into CHO/P cells and examined by an adhesion assay with TK1 cells, a murine T cell lymphoma that expresses high levels of $\alpha_4\beta_7$ (28). Several pools of clones mediated noticeable rosetting of TK1 cells. Binding to one of the pools was completely inhibited by preincubation of TK1 cells with anti-$\beta_7$ mAb Fib 504 (25) (data not shown). This pool was subjected to three rounds of subfractionation until a single clone, called 31D, was isolated. Purified clone 31D mediated $\beta_7$-inhibitable TK1 cell binding (data not shown).

Sequencing of approximately 250 bp of clone 31D revealed an open reading frame that was 53% identical with the first 75 amino acids of murine MAdCAM-1 (see Fig. 4A). In addition to the functional characteristics described above, this sequence had several diagnostic features of a homologue (discussed below) to murine MAdCAM-1. Subsequent to these observations, this cDNA was used to screen a human MLN phage library and isolate a cDNA clone with a similar (~1.7 kb) insert size and almost identical sequence as the primate clone. Transient expression of this cDNA also demonstrated adhesion of TK1 cells that was blocked by anti-$\beta_7$ mAb Fib 504 (25) in addition to inhibition of the human B lymphoblastoid line RPMI 8866 by preincubation with anti-$\alpha_4\beta_7$ mAb Act-1 (26) (not shown). We conclude that these cDNAs encode full-length functional primate and human homologues of MAdCAM-1.

### *Expression of human MAdCAM-1 RNA*

Northern blots, consisting of several human tissues, were probed with the entire human MAdCAM-1 cDNA. A single RNA species of approximately 1.6 kb was highly expressed in the small intestine and expressed to a lesser extent in the colon and spleen (Fig. 1). Expression was absent from several other tissues examined, including thymus, prostate, ovaries, testis, and peripheral blood leukocytes. Other tissues examined were also negative, including heart, brain, placenta, lung, liver, skeletal muscle, and kidney; extremely low expression was observed in the pancreas (not shown).

### *Functional studies*

To determine fully the specificity of human MAdCAM-1-lymphocyte interactions, additional adhesion assays were performed to compare binding of the B cell line RPMI 8866, which expresses $\alpha_4\beta_7$ in the absence of $\alpha_4\beta_1$ (13), with the T cell line Jurkat, which exclusively expresses $\alpha_4\beta_1$. Binding of these cell lines was also compared with that of murine MAdCAM-1 and human VCAM-1. RPMI 8866 cells do not bind control transfectants, but avidly bind both human and murine MAdCAM-1 (Fig. 2A). Binding is completely inhibited by preincubation with anti-$\alpha_4\beta_7$ mAb Act-1. VCAM-1 transfectants fail to bind RPMI 8866. The failure of RPMI 8866s to bind the VCAM-1 transfectants is not due to a lack of expression, as FACS analysis with anti-VCAM-1 mAb 2G7 indicated a transfection efficiency of approximately 60% (not shown). Additionally, these same VCAM-1 transfectants bind Jurkat, and binding is completely inhibited by preincubation with

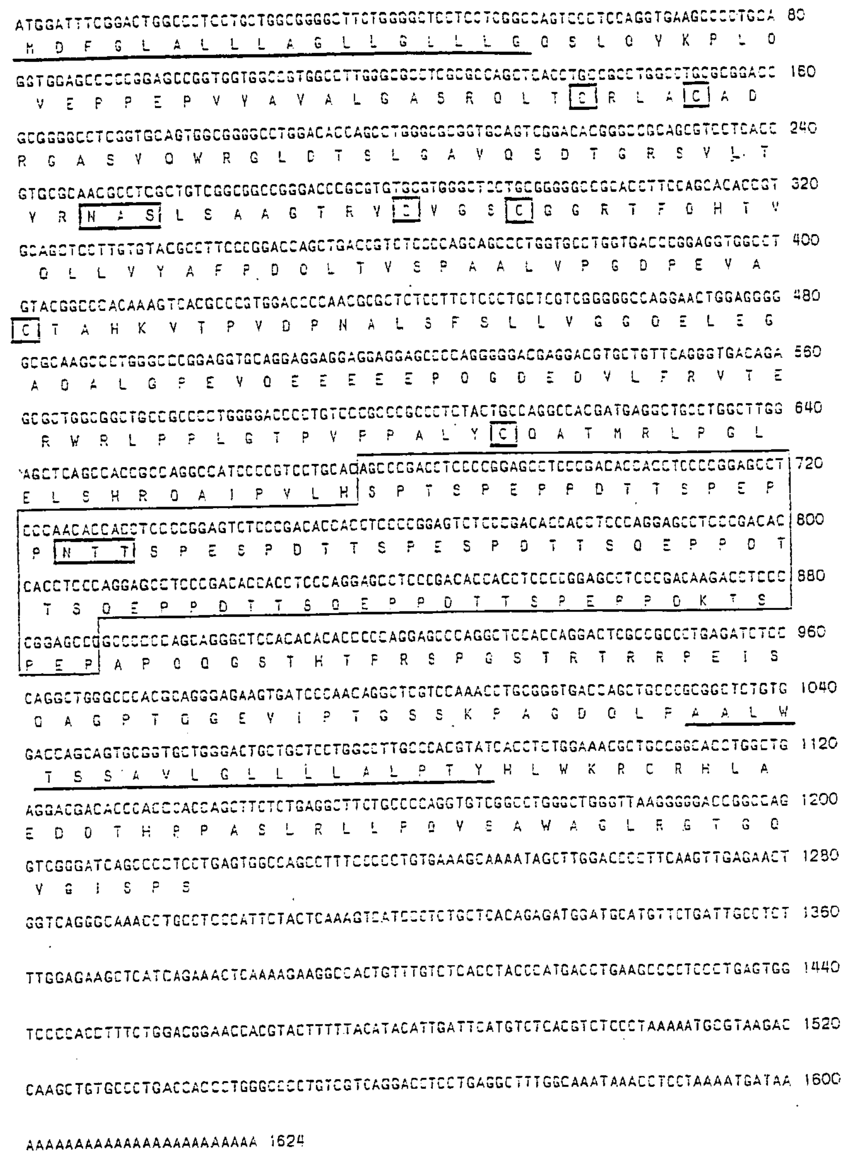

FIGURE 3. Sequence of human MAdCAM-1 cDNA. The signal peptides and transmembrane regions are underlined in bold. Cysteine residues of the two Ig-like domains are boxed, as are potential N-linked glycosylation sites. The repeated portion of the mucin domain with the PPDTTS(Q/P)E motif is outlined.

either anti-VCAM-1 or anti-$\beta_1$ mAbs (Fig. 2*B*), while adhesion of this cell line to murine and human MAdCAM-1 is negative.

### *Sequence analysis*

The cDNA encoding human MAdCAM-1 is 1628 bp (Fig. 3). This clone has no 5′-untranslated region but possesses an open reading frame of 1218 bp encoding a protein of 406 amino acids and a 3′ untranslated region of 410 bp. We believe that the ATG reported is the actual start codon, as this methionine aligns well with the murine sequence (Fig. 4*A*), and analysis of gene structure in murine MAdCAM-1 has shown this ATG to be the initiation codon (29). The mature protein is predicted to be 386 amino acids, for a predicted weight of 40,910 daltons. An L residue at position 15 and a G residue at position 18 are preferred -3 -1 residues for a signal peptide of 18 amino acids (21 amino acids in macaque) (30). Both human and macaque MAdCAM-1 have an identical stretch of 20 hydrophobic residues that aligns with the transmembrane region of murine MAdCAM-1 (Fig. 4*C*). The human MAdCAM-1 cytoplasmic tail is considerably (43 amino acids) longer than those in the murine and macaque (20 and 26 amino acids, respectively) homologues.

The amino-terminal regions of these receptors are similar sequences that comprise the signal peptides and two Ig-like domains (Fig. 4*A*). The remainder of the extracellular domains of human and macaque MAdCAM-1 consist of mucin-like stretches of 117 and 70 amino acids, respectively, that are rich in their content of P/S/T (58 and 50%, respectively) residues (Fig. 4*B*). In contrast, murine MAdCAM-1 has a short mucin region of 37 amino acids (17) that is similar in content of P/S/T residues (51%; Fig. 4*B*). The remainder of the extracellular domain of murine MAdCAM-1 is another predicted Ig domain with homology to IgA. Interestingly, the IgA homology does not occur in the macaque and human clones and is replaced by longer mucin sequences. In the first 71 amino acids of this region in human MAdCAM-1 lies a motif, (P/S)PDTTS(Q/P)E, that is repeated eight times, but is found only once in the murine and macaque homologues (Fig. 4*B*). Therefore, all receptors share mucin-like regions, but there is considerable variation among these homologues. The predicted compared structures of human and murine MAdCAM-1 are shown in Figure 5.

## Discussion

In this study we used a functional cloning approach, relying on the conservation of $\alpha_4\beta_7$ interactions with MAdCAM-1 to isolate a primate MAdCAM-1 homologue that we ultimately used to clone human MAdCAM-1. The lack of nucleotide similarity suggested

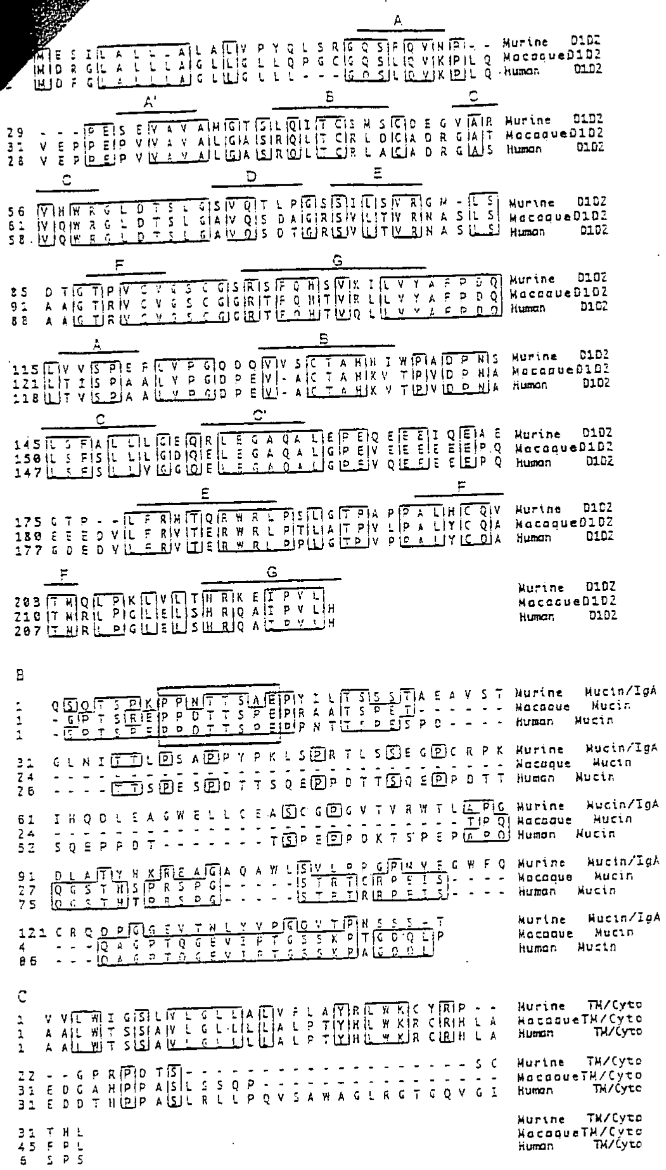

FIGURE 4. Comparison of murine, macaque, and human MAdCAM-1 amino acid sequences. *A*, Alignment of the signal peptides and two N-terminal domains. Highlighted in bold are the cysteine residues and the conserved LDTSL motif involved in binding $\alpha_4\beta_7$. Bars above the alignment represent approximate locations of $\beta$ sheets, as implied by computer programs (Protean, DNASTAR) and the published crystal structure of VCAM-1 (40, 41). *B*, Alignment of mucin sequences of human and maqaque MAdCAM-1 with the mucin IgA domain of murine MAdCAM-1. The first row of the alignment shows (boxed in bold) the eight-amino acid sequence that aligns only once in the three receptors but is repeated eight times (also in bold) in human MAdCAM-1. *C*, Alignment of the transmembrane and cytoplasmic domains. Sequence alignments were performed by the Cluster method using a PAM 250 weight table, a gap penalty of 10, and a gap length penalty of 10. Pairwise alignment parameters were: ktuple = 1, gap penalty = 3, window = 4 and diagonals saved = 5.

by our previous studies was confirmed by sequence comparisons. The overall nucleotide sequence identity between human and murine MAdCAM-1 is poor at 42%, while the protein identities are even weaker at 39%. Homology searches of multiple databases indicate that human MAdCAM-1 is a unique sequence whose only significant homology is (as reported with murine MAdCAM-1 (17)) to VCAM-1 and ICAM-1.

RNA transcripts hybridizing to human MAdCAM-1 cDNA were detected only in small and large intestine and the spleen, while several other tissues probed were negative (Fig. 1). This pattern is consistent with studies in the mouse showing restricted expression of MAdCAM-1 in PP, MLN, and intestinal lamina propria and some expression in the marginal sinus around splenic white pulp nodules in the spleen (7, 8, 17). These results indicate that human MAdCAM-1 is primarily expressed in mucosal tissues, although a thorough analysis of its tissue distribution in both normal and inflamed tissues will require immunohistochemistry with mAbs currently being developed.

Human MAdCAM-1 avidly binds RPMI 8866, a human B cell line that expresses $\alpha_4\beta_7$, while VCAM-1 fails to bind this cell line (Fig. 2*A*). Conversely, the $\alpha_4\beta_1$ high T cell line, Jurkat, binds only VCAM-1. These results are consistent with previous observations that $\alpha_4\beta_7$-VCAM-1 interactions are activation dependent (12, 32, 33), while MAdCAM-1 binds $\alpha_4\beta_7$ in the absence of activation. Recently, we have also shown that human lymphocyte subsets expressing high levels of $\alpha_4\beta_7$ also preferentially bind murine MAdCAM-1 (see Footnote 4). While these studies demonstrate that $\beta_7^{high}$ $CD4^+$ memory cells can also bind VCAM-1, adhesion of these cells is inhibited by blockade with an anti-$\beta 1$ mAb. These studies along with the adhesion assays in this report reinforce that the selectivity of $\alpha_4\beta_7$ as the ligand for MAdCAM-1 in mucosal sites has been retained in humans.

While the MAdCAM-1 homologues are extremely divergent, sequence comparisons demonstrate that the first two (N-terminal) Ig-like domains, at 57% identity, are the most highly conserved regions of these receptors (Fig. 4*A*) (5). The two Ig loops display several features conserved with murine MAdCAM-1, including: 1) double cysteine residues, separated by three amino acids in the first Ig domain; 2) an identical nine-amino acid stretch in Ig domain 1 (Fig. 4*A*), containing the sequence LDTSL, which aligns with a consensus motif for integrin-Ig family member interactions (34–38); and 3) a uncharacteristically large second Ig domain, with approximately 70 amino acids between cysteine residues. This is a novel feature in comparison to other Ig-like adhesion receptors, which usually have 40 to 50 residues between the cysteines in each domain (39, 40).

Although the LDTSL motif in domain 1 has general conservation with respect to other Ig adhesion receptors, such as ICAM-1, ICAM-2, ICAM-3, and VCAM-1 (33–37), this exact sequence has only been found in murine MAdCAM-1 (17) (Fig. 4*A*). The functional significance of this motif has been demonstrated by the fact that a point mutation in amino acid 61 that changed the first L to an R in this sequence had a dramatic effect on $\alpha_4\beta_7$ binding to murine MAdCAM-1 (18). Recent crystallographic analysis of the two N-terminal domains of VCAM-1 indicate that the analogous sequence, QIDSPL, resides in the highly exposed C-D loop of Ig domain 1 and is thus free to interact with its integrin ligand (40, 41). The conservation of this sequence in addition to studies showing that it can be exchanged with its homologous sequence in ICAM-1 with no change in function suggest that this motif is a general integrin recognition element (34). Within domain 2 of VCAM-1 is a highly extended C′-E loop that has a similar sequence which is even more accentuated in all species of MAdCAM-1 clones. This proposed human MAdCAM-1 C′-E loop, which consists of nine negatively charged (D or E) residues, is 19 amino acids in length compared with 10 to 12 amino acids (40, 41) in VCAM-1 (Fig. 4*A*). While the C′-E loops of the MAdCAM-1 clones are not identical, the length and the highly charged character of this region are well conserved (Fig. 4*A*). The

BEST AVAILABLE COPY

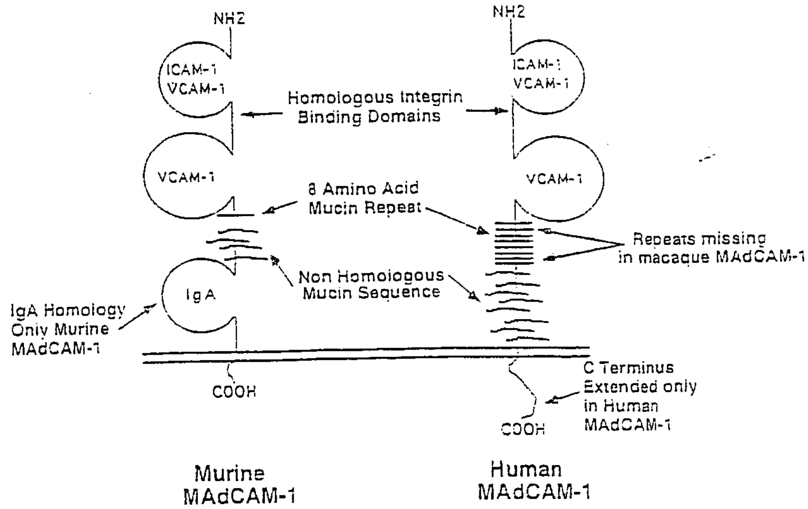


FIGURE 5. Compared structures of murine and human MAdCAM-1. The differences in the structures of macaque and human MAdCAM-1 are also indicated.

crystal structure of VCAM-1 shows that this loop is also highly exposed and in close proximity to the CD loop of domain 1 and, along with its unique features, may contribute to the specificity of integrin binding.

The mucin regions are the most divergent sequences in the MAdCAM-1 homologues (Fig. 4*B*). Extensive polymorphism and sequence divergence have been documented in other mucin-like sequences as well. Episialin, for example, contains a 20-amino acid repeat that can vary in copy number from 30 to 90 (43, 44), while repetitive portions of intestinal mucins are not well conserved between rodents and humans (45). It is of interest to speculate on the capability of these divergent sequences to serve as substrates for L-selectin binding carbohydrates. No specific sequence requirement has been identified with this modification, as GLYCAM-1, CD34, and murine MAdCAM-1 all bind L-selectin, but are only related by their high content of S/T/P residues (17, 19, 46, 47). It is, therefore, likely that these variant sequences in the MAdCAM-1 homologues described are all capable of presentation of this carbohydrate structure. An alternative possibility, however, is that there is post-transcriptional processing to generate variants of the cDNAs isolated that might differ in their abilities to bind L-selectin. Along these lines, we have recently described a alternatively processed form of murine MAdCAM-1 that lacks the mucin/IgA domain (30) and is thus proposed to lack the capability to bind L-selectin. mAbs to the human receptor will allow us to examine the carbohydrate modifications of human MAdCAM-1 and their contribution to binding L-selectin.

In summary, human MAdCAM-1 has retained sequences important for selectively binding the $\alpha_4\beta_7$-integrin, whereas mucin-like sequences have greatly diverged. While most closely related to VCAM-1, cell adhesion studies in this report reinforce previous data that demonstrate that these two receptors are functionally distinct. The cloning of these MAdCAM-1 homologues will assist in additional sequence comparisons and identification of amino acid residues that may be critical for binding to $\alpha_4\beta_7$. mAbs to human MAdCAM-1 will also facilitate an examination of the expression of this gut-associated addressin in normal and inflamed tissues, such as inflammatory bowel disease.

## Acknowledgments

The authors thank Walter Newman and Eugene C. Butcher for helpful discussions and comments, and Doug Ringler for his contribution of valuable tissues for cDNA library construction.

## References

1. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell 67:1033.*
2. Bevilacqua, M. P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol. 11:767.*
3. Springer, T. A. 1994. Traffic signals for lymphocyte and leukocyte emigration: the paradigm. *Cell 76:301.*
4. Berg, E. L., L. J. Picker, M. K. Robinson, P. R. Streeter, and E. C. Butcher. 1991. Vascular addressins: tissue selective endothelial adhesion molecules for lymphocyte homing: cellular and molecular mechanisms of inflammation. *2:111.*
5. Picker, L. J., and E. C. Butcher. 1992. Physiological and molecular mechanisms of lymphocyte homing. *Annu. Rev. Immunol. 10:561.*
6. Nakache, M., E. Lakey-Berg, P. R. Streeter, and E. C. Butcher. 1989. The mucosal vascular addressin is a tissue-specific endothelial adhesion molecule for circulating lymph nodes. *Nature 337:179.*
7. Streeter, P. R., E. Lakey-Berg, B. T. N. Rouse, R. F. Bargatze, and E. C. Butcher. 1988. A tissue-specific endothelial cell molecule involved in lymphocyte homing. *Nature 331:41.*
8. Sikorski, E. E., R. Hallmann, E. L. Berg, and E. C. Butcher. 1993. The Peyer's patch high endothelial receptor for lymphocytes, the mucosal addressin, is induced on a murine endothelial cell line by tumor necrosis factor-α and IL-1. *J. Immunol. 151:5239.*
9. Faveeuw, C., M.-C. Gagnerault, and F. Lepault. 1994. Expression of homing and adhesion molecules in infiltrated islets of Langerhans and salivary glands of nonobese diabetic mice. *J. Immunol. 152:5969.*
10. Hanninen, A., C. Taylor, P. R. Streeter, L. S. Stark, J. M. Sarte, J. A. Shizuru, O. Simell, and S. A. Michie. 1993. Vascular addressins are induced on islet vessels during insulitis in nonobese diabetic mice and are involved in lymphoid cell binding to islet endothelium. *J. Clin. Invest. 92:2509.*
11. O'Neill, J. K., C. Butter, D. Baker, S. E. Gschmeissner, G. Kraal, E. C. Butcher, and J. L. Turk. 1991. Expression of vascular addressins and ICAM-1 by endothelial cells in the spinal cord during chronic relapsing experimental allergic encephalomyelitis in the Biozzi AB/H mouse. *Immunology 72:520.*
12. Berlin, C., E. L. Berg, M. J. Briskin, D. P. Andrew, P. J. Kilshaw, B. Holzmann, I. L. Weissman, A. Hamann, and E. C. Butcher. 1993. $\alpha_4\beta_7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell 74:185.*
13. Erle, D. J., M. J. Briskin, E. C. Butcher, A. Garcia-Pardo, A. I. Lazarovits, and M. Tidswell. 1994. Expression and function of the MAdCAM-1 receptor, integrin $\alpha_4\beta_7$, on human leukocytes. *J. Immunol. 153:517.*
14. Hamann, A., D. P. Andrew, D. Jablonski-Westrich, B. Holzmann, and E. C. Butcher. 1994. The role of $\alpha_4$ integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol. 152:3282.*
15. Salmi, M., D. P. Andrew, E. C. Butcher, and S. Jalkanen. 1995. Dual binding capacity of mucosal immunoblasts to mucosal and synovial endothelium in humans: dissection of the molecular mechanisms. *J. Exp. Med. 181:137.*
16. Schweighoffer, T., Y. Tanaka, M. Tidswell, D. J. Erle, K. J. Horgan, G. E. G. Luce, A. I. Lazarovits, D. Buck, and S. Shaw. 1993. Selective expression of integrin $\alpha_4\beta_7$ on a subset of human CD4⁺ memory T cells with hallmarks of gut-tropism. *J. Immunol. 151:717.*
17. Briskin, M. J., L. M. McEvoy, and E. C. Butcher. 1993. MAdCAM-1 has homology to immunoglobulin and mucin-like adhesion receptors and to IgA1. *Nature 363:461.*
18. Briskin, M. J., L. S. Rott, and E. C. Butcher. 1996. Structural requirements for mucosal vascular addressin binding to its lymphocyte receptor α4β7: common themes among integrin-Ig family interactions. *J. Immunol. 156:719.*
19. Berg, E. L., L. M. McEvoy, C. Berlin, R. F. Bargatze, and E. C. Butcher. 1993. L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature 366:695.*

BEST AVAILABLE COPY

...an, C., R. F. Bargatze, J. J. Campbell, U. H. von Andrian, M. C. Szabo, S. R. Hassien, R. D. Nelson, E. L. Berg, S. L. Erlandsen, and E. C. Butcher. 1994. $\alpha_4$ integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell 80:413.*

21. Bargatze, R. F., M. A. Jutila, and E. C. Butcher. 1995. Distinct roles of L-selectin and integrins $\alpha_4\beta_7$ and LFA-1 in lymphocyte homing to Peyer's patch-high endothelium in situ: the multi-step hypothesis confirmed and refined. *Immunity 3:99.*
22. Osborn, L., C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell 59:1203.*
23. Polte, T., W. Newman, and T. Venkat Gopal. 1990. Full-length vascular cell adhesion molecule-1. *Nucleic Acids Res. 18:5901.*
24. Graber, N., T. Venkat Gopal, D. Wilson, L. D. Beall, T. Polte, and W. Newman. 1990. T cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotein and endothelial leukocyte adhesion molecule-1. *J. Immunol. 145:819.*
25. Andrew, D. P., C. Berlin, S. Honda, T. Yoshino, A. Hamann, B. Holzmann, P. J. Kilshaw, and E. C. Butcher. 1994. Distinct but overlapping epitopes are involved in $\alpha_4\beta_7$-mediated adhesion to vascular cell adhesion molecule-1, mucosal addressin-1, fibronectin, and lymphocyte aggregation. *J. Immunol. 153:3847.*
26. Lazarovits, A. I., R. A. Moscicki, J. T. Kurnick, D. Camerini, A. K. Bhan, L. G. Baird, M. Erickson, and R. E. Colvin. 1984. Lymphocyte activation antigens. I. A monoclonal antibody, ACT-I, defines a new lymphocyte activation. *J. Immunol. 133:1857.*
27. Heffernan, M., and J. D. Dennis. 1991. Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells. *Nucleic Acids Res. 19:85.*
28. Butcher, E. C., R. G. Scollay, and I. L. Weissman. 1980. Organ specificity of lymphocyte interaction with organ specific determinants on high endothelial venules. *Eur. J. Immunol. 10:556.*
29. Wu, N. W., S. Jalkanen, P. R. Streeter, and E. C. Butcher. 1988. Evolutionary conservation of tissue-specific lymphocyte-endothelial cell recognition mechanisms involved in lymphocyte homing. *J. Cell Biol. 107:1845.*
30. Sampaio, S. O., X. Li, M. Takeuchi, C. Mei, U. Francke, E. C. Butcher, and M. B. Briskin. 1995. Organization, regulatory sequences, and alternatively spliced transcripts of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) gene. *J. Imunol. 155:2477.*
31. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res. 14:4683.*
32. Postigo, A. A., P. Sanchez-Meyeos, A. I. Lazarovits, F. Sanchez-Madrid, and M. O. de Landazuri. 1993. $\alpha_4\beta_7$ integrin mediates binding to fibronectin and vascular cell adhesion molecule-1. *J. Immunol. 151:2471.*
33. Ruegg, C., A. Postigo, E. Sikorski, E. C. Butcher, R. Pytela, and D. J. Erle. 1992. Role of integrin $\alpha_4\beta_7/\alpha_4\beta_p$ in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J. Cell Biol. 117:179.*
34. Osborn, L., C. Vassallo, B. G. Browning, R. Tizard, D. O. Haskard, C. D. Benjamin, I. Dougas, and T. Kirchhausen. 1994. Arrangement of domains, and amino acid residues required for binding of vascular cell adhesion molecule-1 to its counter-receptor VLA-4 ($\alpha_4\beta_1$). *J. Cell Biol. 124:601.*
35. Renz, M. E., H. H. Chiu, L. Fox, K. J. Kim, L. G. Presta, and S. Fong. 1994. Structural requirements for adhesion of soluble recombinant murine vascular cell adhesion molecule-1 to $\alpha_4\beta_1$. *J. Cell Biol. 125:1395.*
36. Staunton, D. E., M. L. Dustin, H. P. Erickson, and T. A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell 61:243.*
37. Vonderheide, R. H., T. F. Tedder, T. A. Springer, and D. Stuanton. 1994. Residues within a conserved amino acid motif of domains 1 and 4 of VCAM-1 are required for binding to VLA-4. *J. Cell Biol. 125:215.*
38. Holness, C. A., P. A. Bates, A. J. Littler, C. D. Buckley, A. L. McDowall, D. Bossey, N. Hogg, and D. L. Simmons. 1995. Analysis of the binding site on intracellular adhesion molecule 3 for the leukocyte integrin lymphocyte function-associated antigen-1. *J. Biol. Chem. 270:877.*
39. Hunkapiller, T., and L. Hood. 1989. Diversity of the immunoglobulin gene superfamily. *Adv. Immunol. 44:1.*
40. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol. 6:381.*
41. Jones, E. Y., K. Harlos, M. J. Bottomley, R. C. Robinson, P. C. Driscoll, R. M. Edwards, J. M. Clements, T. J. Dudgen, and D. I. Stewart. 1995. Crystal structure of an integrin-binding fragment of vascular cell adhesion molecule-1 at 1.8 A resolution. *Nature 373:539.*
42. Wang, J.-H., R. B. Pepinsky, T. Steele, J.-H. Liu, M. Karpusas, B. Browning, and L. Osborn. 1995. The crystal structure of an N-terminal two-domain fragment of vascular cell adhesion molecule 1 (VCAM-1): a cyclic peptide based on the domain 1 C-D loop can inhibit VCAM-1-$\alpha_4$ interaction. *Proc. Natl. Acad. Sci. USA 92:5714.*
43. Hilkens, J. M., J. L. Ligtenberg, H. L. Vos, and S. V. Litvinov. 1992. Cell membrane-associated mucins and their adhesion-modulating property. *Trends Biochem. Sci. 17:359.*
44. Jentoft, N. 1990. Why are proteins O-glycosylated? *Trends Biochem. Sci. 15:291.*
45. Gum, J. G., J. W. Hicks, R. E. Lagace, J. C. Byrd, N. W. Toribara, B. Siddiki, F. J. Fearney, D. T. A. Lamport, and Y. S. Kim. 1991. Molecular cloning of rat intestinal mucin. *J. Biol. Chem. 266:22733.*
46. Baumhueter, S., M. S. Singer, W. Henzel, S. Hemmerich, M. Renz, S. D. Rosen, and L. A. Lasky. 1993. Binding of L-selectin to the vascular sialomucin, CD34. *Science 262:436.*
47. Lasky, L. A., M. S. Singer, D. Dowbenko, Y. Imai, W. J. Henzel, C. Grimley, C. Fennie, N. Gillett, S. R. Watson, and S. D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell 69:927.*


BEST AVAILABLE COPY